Il processo di una scoperta
scientifica è, in effetti, un
continuo conflitto di meraviglie.
Albert Einstein

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BASEMENT MEMBRANE AND CELLULAR MIGRATION: ROLE OF GELATINASES (MMP-2, MMP-9) ON PROTEOLYSIS OF TYPE IV COLLAGEN

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Abstract

Proteolytic degradation of basement memnbrane influences the cell behaviour during important processes, such as inflammations, tumorigenesis, angiogenesis and allergic diseases. In this study, we have investigated the action of gelatinase A (MMP-2) and B (MMP-9) on collagen IV, the major constituent of the basement membrane. We have compared quantitatively their actions on the soluble forms of collagen IV extracted with or without pepsin (from human placenta and from Engelbreth-Holm-Swarm (EHS) murine sarcoma, respectively). The catalytic efficiency of MMP-2, and also MMP-9, is dramatically reduced in the case of the EHS murine sarcoma with respect to the human placenta, probably due to the much tighter packing of the network which renders very slow the speed of the rate-limiting step.

We have also enquired on the role of MMP-2 domains in processing collagen IV. The removal of the hemopexin-like domain, using only the catalytic domain of MMP-2, has only a limited effect on the catalytic efficiency toward collagen IV, indicating that the missing domain has not a great relevance for the overall mechanism. Instead, the addition of the isolated collagen binding domain, corresponding to the fibronectin-like domain of whole MMP-2, greatly inhibits the cleavage process, demonstrating that MMP-2 interacts with collagen type IV preferentially through its fibronectin-like domain. Finally, we have investigated the effect of MMP-2 proteolytic activity *ex vivo*. MMP-2 action negatively affects the neutrophil cells migration across type IV coated membranes and this is likely related to the production of lower molecular weight fragments which impair the cellular migration.However, for both types of collagen IV the enzymatic processing by MMP-9 is dramatically enhanced in the presence of the Collagen Binding Domain of Gelatinase A (CBD). This effect, clearly indicates that the fibronectin-like domain of MMP-2 and MMP-9 bind to topologically distinct sites on type IV collagen, bringing about a conformational change of the collagen IV molecule. This allows the two enzymes to cooperate with each other through a ligand-linked mechanism, which does not necessarily require the enzymatic action. Therefore, fibronectin-like domains not only increase the affinity between enzyme and substrate to enhance the catalysis, they also act as allosteric third party elements in the MMP action. This synergistic action between MMP-2 and MMP-9 on collagen IV has been tested also with an ex vivo eperiment. The MMP-2 without the catalytic domain, the rCBD and the pro-MMP-2 increase the neutrophil cells migration across collagen IV coated membranesand this is related to the growth of the catalytic activity of MMP-9.

Riassunto

In condizioni patologiche le gelatinasi o meglio la MMP-2 e la MMP-9 vengono espresse dalle cellule in quantità più elevate rispetto alle normali condizioni fisiologiche. Ne sono un esempio i processi infiammatori e la progressione tumorale, dove queste endopeptidasi Ca^{+2} e Zn^{+2} dipendenti sono attivamente coinvolte nel processamento delle membrane basali cellulari; infatti la loro azione permette alle cellule tumorali la loro progressione all'interno di nuovi tessuti, dando origine alla formazione di metastasi. Le gelatinasi influenzano la progressione tumorale in quanto intervengono in un processo che prende il nome di "angiogenic switch", che consiste nella formazione di una nuova vascolarizzazione in grado di alimentare le cellule in attiva proliferazione. L' "angiogenic switch" inizia con la degradazione da parte delle gelatinasi del collagene IV, componente fondamentale della membrana basale vascolare. Esso, una volta processato, libera fattori (quali il VEGF) che stimolano le cellule endoteliali a migrare all'interno di una matrice provvisoria dove si formeranno i nuovi vasi sanguigni. Se da un lato le gelatinasi aumentano l'attività proliferativa delle cellule tumorali, dall'altra sono in grado di inibirla. Sembra infatti che la MMP-2, degradando il collagene di tipo IV, produca dei frammenti con attività anti-angiogenica in grado di bloccare la proliferazione tumorale.
Ai fini di una miglior comprensione del ruolo delle Gelatinasi durante la progressione tumorale, abbiamo analizzato l'efficienza delle due metalloproteinasi verso il collagene di tipo IV; e nel caso della MMP-2 abbiamo caratterizzato il ruolo dei suoi diversi domini verso questo substrato naturale. Inoltre è stata effettuata una analisi basata sulla capacità dei neutrofili di migrare attraverso una membrana ricoperta di collagene di tipo IV in presenza di (i) una quantità esogena crescente di MMP-2 (ii) frammenti di arrestina derivanti dal processamento della catene α del collagene IV, (iii) MMP-2 mutata, ossia priva del suo dominio catalitico, (iv) del dominio ricombinante fibronectinico della MMP-2 o rCBD e (v) la MMP-2 inattiva o pro-MMP-2.Per finire abbiamo studiato l'esistenza di una possibile interazione tra le due gelatinasi nel processare il collagene di tipo IV sia in vitro che in condizioni ex vivo.I nostri risultati dimostrano come entrambe le gelatinasi processano il componente fondamentale delle membrane basale vascolare in condizioni fisiologiche, 37°C e pH 7,2. Inoltre l'MMP-2 interagisce con la MMP-9 aumentandone l'efficienza di processamento verso il collagene di tipo IV, per effetto di un controllo allosterico dovuto al semplice legame dell'enzima con il substrato. Quando la MMP-2 si lega al collagene IV per opera del suo dominio fibronectinico (CBD) , la MMP-9 processa il substrato con maggiore efficienza rispetto all'

azione quando è prsente da sola. Il fenomeno varia a seconda della concentrazione della MMP-2 che possiede due siti di legame per questo tipo di collagene: uno ad alta affinità ed un secondo sito con una scarsa affinità di legame. A bassa concentrazione di MMP-2 viene legato solo il sito del substrato per cui l'enzima posside una maggior affinità e di conseguenza la MMP-9 legandosi allo stesso substrato lo processa con maggior efficienza. Al contrario, ad alte concentrazioni di MMP-2, l'enzima si lega ad ambedue i siti di attacco che possiede nel collagene IV diminuendo la capacità della MMP-9 di processarlo, a causa di una probabile sovrapposizione del sito di attacco dei due enzimi.L'attività sinergica delle due gelatinasi viene riscontrata anche *ex vivo*, ossia mediante esperimenti di migrazione cellulare attraverso un coating di collagene IV. Le cellule utilizzate sono neutrofili, cellule in grado di esprimere vari enzimi proteolitici tra cui metalloproteinasi come la MMP-8 e la MMP-9 e proteasi a serina come l'elastasi del neutrofili. Risultano invece non produrre livelli apprezzabili di MMP-2, come dimostrato dalle zimografie delle Figure 10 e 11. I neutrofili, grazie alla MMP-9, sono quindi in grado di oltrepassare la barriera di collagene IV, che viene processato con maggior attività in presenza di quantità esogene di MMP-2 priva del dominio catalitico o in aggiunta del suo dominio di legame al substrato, il dominio ricombinante rCBD.

Introduction

I) Basement Membranes: role in tumour angiogenesis

In recent yers, the basement membrane (BM), a specialized form of the extracellular matrix (ECM), has been recognized as an important regulator of cell behaviour. The importance of BM becomes relevant when some vascular diseases have been known to be associated with defects on BM components. Diseases such as Alport syndrome and Knoblach syndrome have been associated to mutational defects in type IV of collagen and α chainsof collagen type XVIII.

1) Basement membrane structure and assembly

The basement membrane is an amorph, dense sheet-like structure of 50-100 nm in thickness that generally separates ephitelium from the stroma of any given tissue. It is always in contact with cells, and its function is to provide structural support, todivide tissue into compartments, as well as to regulate cell behaviour.
BMs are highly crosslinked and insoluble material that are composed of several glycoproteins.

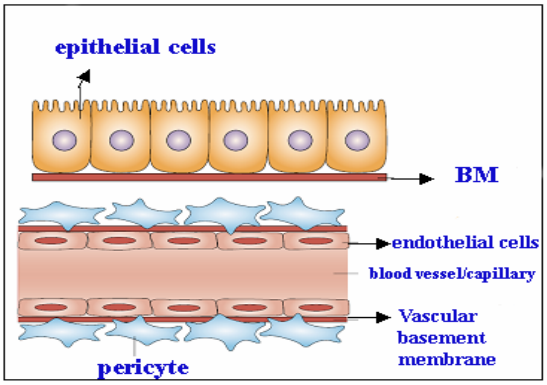


Figure 1: Basement membrane and vascular basement membrane localization.
Different structure and localization of the basement membrane (BM) on the top of the figure and of the vascular basement membrane or VBM.

a) Basement membrane glycoproteins

The costituens of BM are usually insoluble molecules that come together to form sheet-like structures via a process known as self-assembly which is driven by cell surface receptors. In general, all cells are known to produce the BM components; some of them are different kinds of collagen, proteoglycans, laminin polymers and nidogen/entactin complexes. Minor components are agrin, SPARC/BM-40/osteopontin, fibulins (Figure 2).

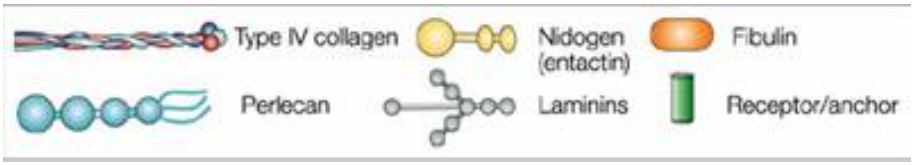


Figure 2: Glycoprotein composition of the BM structure. Type IV of collagen protomers, nidogen/entactin complex, fibulin, perlacan, laminin polymers.

a1) Collagen

Collagens, are a class of proteins that are carachterized by a unique sequence stretch in wich every third amino acid is a Glycine (GLY) (Gly-X-Y) motif. The X and Y motifs are usually hydroxyproline and hydroxylysine. Type I of collagen along with type II and type III collagen are classic examples of proteins that have long stretches of the Gly-X-Ymotif and are termed fibrillar collagen for their capacity to form fibres.

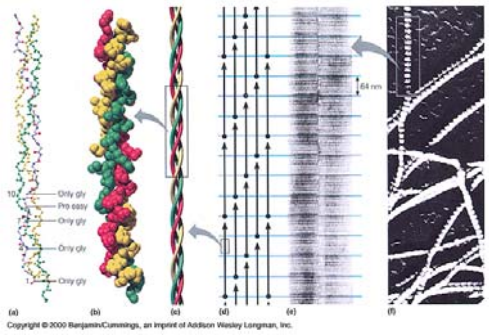


Figure 3. Fibrillar collagen. a) b) the molecular structures of the collagenous proteins depicted with ball-and-stick models c) schematic representation of triple helix d) e) 67 nm D periodic fibrils f) electron microscopy of intact calf skin collagen fibrils.

Collagens have the capacity to self-associate in different forms. At least 15 other types of collagens have been identified, which are not homologous to the fibril forming collagens, and can adopt other supramolecular organizations. These non-fibrillar collagen types include the network assembly of type IV collagen in basement membranes, the antiparallel associations of type VII collagen in anchoring fibrils, and the hexagonal arrays of type VIII collagen in Descemet's membrane (Kramer et al., 2001)
The collagen super family is classified into groups according to the polymeric structures they form or to related structural features:

a) fibril forming collagens (types I, II, III, V and XI)
b) network forming collagens (type IV, VIII and X)
c) collagens that are found on the surface of fibrils known as fibril-associated collagens with interrupted triple helices (FACIT) (types IX, XII, XVI and XIX)
d) beaded filament-forming collagen (type VI)
e) collagen of anchoring fibrils for basement membranes (type VII)
f) collagens with a transmembrane domain (type XIII and XVII)
g) family of type XV and XVIII
h) "noncollagen" collagen consists of proteins containing triple-helical domains that have not been defined collagens (subcomponent C1q of complement (Prockop et al., 1995).

The BM contains type IV, XV and XVIII collagen. Type IV of collagen is the most abundant component of vascular basement membrane (VBM) and it is also called network-forming collagen due to its capacity to self assemblate into organized network. It is generally formed by the association of three different α-chains in a primary structure called protomer. Six different type IV of collagen α chains have been identified codified by six different genes.
Type XV collagen is widely distributed in several BM zones of various tissue, it is a non-fibrillar collagen and it is thought to be a homotrimer formed by three α1 chains. It is a highly glycosylated collagen with a central triple helical domain that is interrupted by NC domains and the protein contains large amino terminal and carboxy terminal NC domains. Expression of type XV of collagen is highest in the heart and skeletal tissue. It is highly

homologous to type XVIII of collagen for the similarity of the NC terminal domain. This kind of collagen is particulary important for skeletal muscular stability and in mouse deficient for this collagen we show a normal capillary structure.
Type XVIII collagen is a component of several different types of ephitelial and vascular BMs, is constituted by different domains with globular and thriple helical structure and it is expressed as three variants that have differences in their amino-terminal regions. Its carboxy terminal domain (NC) contains a fragment called endostatin with an anti angiogenic activity. Type XVIII collagen is considered important for the normal development of the vasculature in the retina. Infact a splice mutation in human collagen XVIII has been associated with Knobloch syndrome, a disease in which the retinal vasculature fails to develop and atients suffer among other abnormalities retinal degeneration and blindness (Kalluri, 2003).

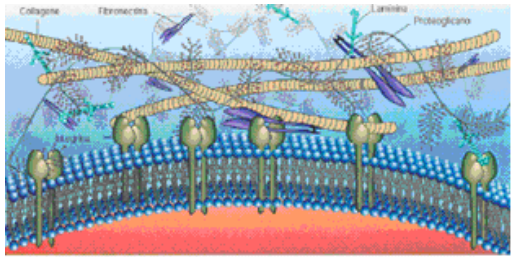


Figure 4: BM glycoproteins. The basement membrane is carhacterized by the interaction of collagens and different glycoproteins such as fibronectin, laminin and proteoglycans.

a2) other glycoproteins

Laminin was the first noncollagenous glycoprotein purified from tumour material, and it is one of the most potent cell adhesion molecules .

It contains different binding sites specific for cells, proteoglycans and collagens. It is very important for the binding of ephitelial cells to the basement membrane. It has been shown that there are tissue specific isoforms of laminin; and laminins are large disulfide-bonded heterotrimers composed of three genetically distinct polypeptide chains, α, β and γ, whose genes are located on different chromosomes. The best characterized laminin molecule, laminin 1 was isoleted from the Engelbreth-Holm-Swarm tumour. It contains one 400 kDa chain, the α1 chain, and two 200 kDa chains, the β1 and γ1 chains, which associate to form a cross shaped molecule.Variants of the α (α2, α3,α4), β (β2, β3) , and γ (γ2) chains were identified, and there are indications that additional chains exist as well.Association of one α, one β and one g chain into various heterotrimers determines the existence of laminin isoforms.These isoforms differ from lamini 1 by one chain like laminin 2 (α2β1γ2) or laminin 3 (α1β2γ1), two chains like laminin 4 (α2β2γ1), or three chains like laminin 5 (α3β3γ2).

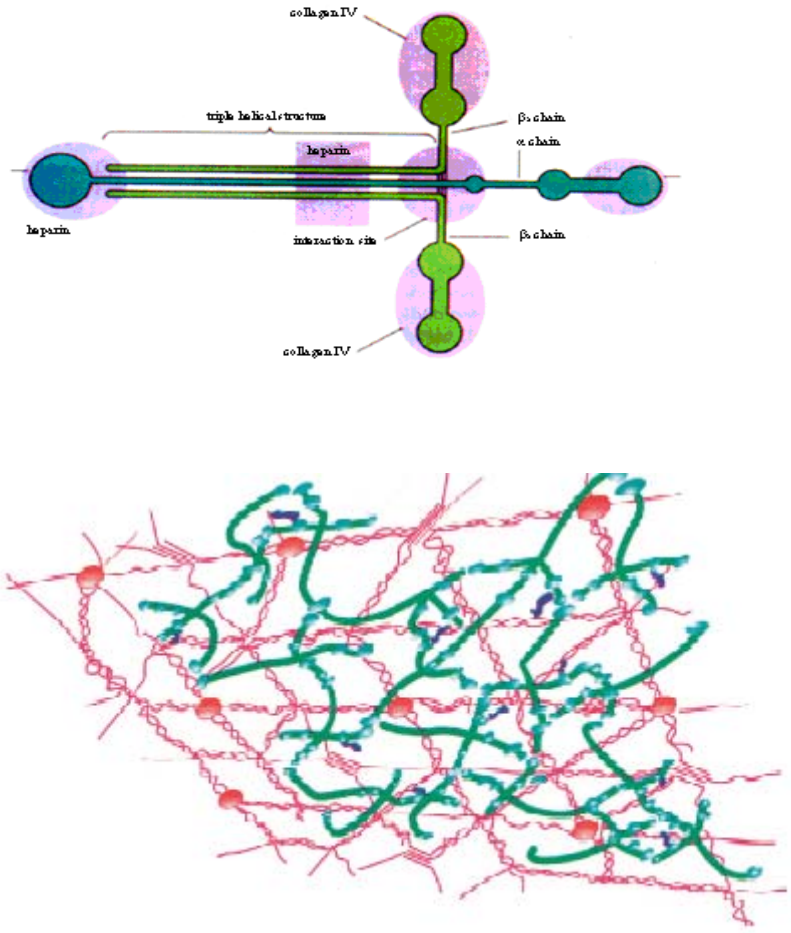


Figure 5: Laminin structure and type IV of collagen network on basement membrane.

Enactin and nidogen are different names for the same compound. This glycoprotein is approximately 150 kDa and consists of three globular domains separeted by two linear segments.It has been linked to a dumb-bell in shape. It is highly susceptible to proteolysis and has both matrix and cell binding propreties.Enactin/nidogen complex binds tightly to laminin via the γ1 chain. Collagen IV also binds to enactin/nidogen. These interaction occur via the carboxyl-terminal globule of enactin/nidogen. It is not clear if this complex has a role other than as a structural stabilizer in the GBM (Levidiotis and A Powe, 2005).

Proteoglycans are found in all basement membranes. They are a class of molecules characterized by a protein core covalently linked to at last one glycosaminoglycan side-chain. The functions of proteoglycans are due to their glycosaminoglycan side-chains. Glycosaminoglycans are unbranched anionic polymers of either glucose-glucose or galactose-glucose derived disaccharides. Heparan sulphates are complex structures belonging to the glycosaminoglycan family. They consist of alternating ialuronic acid and glucosamine residues modified at various positions by sulphation, epimerization or N-acetylation.Proteoglycans are expressed on the surface of most cell types and are important in cell adhesion to the extracellular matrix, cell-cell interactions, and in the interactions of soluble growth factors with their cell surface receptors. Heparan sulphate bound to structural proteoglycans also bind growth factors and other cytokines. Growth factors including platelet-derived growth factor (PDGF), trasforming growth factor-β (TGF-b), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) are bound by heparan sulphate proteoglycans (HSPG).

Perlecan is a large multidomain extracellular matrix proteoglycan that has been conserved in organisms. It is expressed in nearly all basement membranes and connetive tissues. The protein core consists of five domains and numerous sites for O-linked glycosylation as well as four potential sites for heparan sulphate chain attachment: three on domain I and one on domain V. These carbohydrate chains, as well as the domains of protein core, are known to interact with a wide range of biological molecules (Knox and Whitelock, 2006).

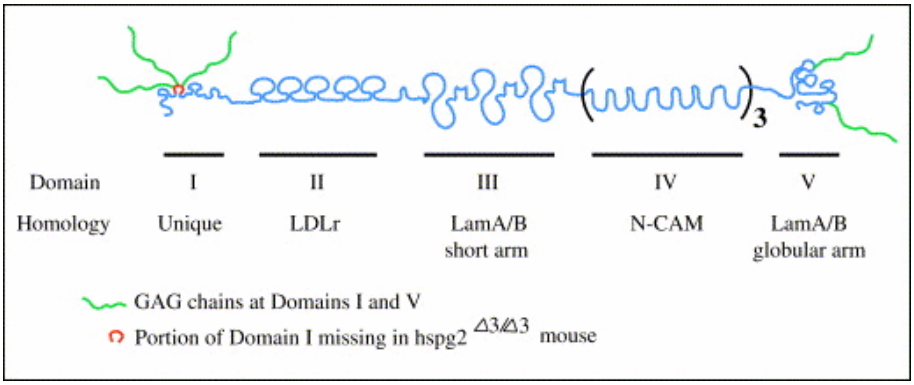


Figure 6: Perlecan core protein: determined domain structure and GAG attachment. Domains II–V of perlecan show homology to the LDL receptor (LDLr), the short arm of laminins A and B, the neural cell adhesion molecule (N-CAM), and the globular arm of laminins A and B. The heparan sulfate or chondroitin sulfate GAGs are attached on domains I and V. Also depicted is the region of domain I that is absent when exon 3 is disrupted (Smith and Hassel, 2006).

The core protein of perlecan (with its globular domains spaced by rod-like sequences) resembles a string of pearls, hence its name . The protein core is encoded by an approximately 120 kb gene with 97 exons . The gene is well conserved across species, with homologues of mammalian perlecan present in Caenorhabditis elegans , Drosophila and other species. In humans, perlecan's core protein is 466 kDa in size. Mice also have a 369 kDa core protein due to alternative splicing .

Fibulins are a family of five extracellular matrix glycoproteins of a fibulin-type C-terminal domain preceded by tandem calcium binding epidermal growth factor like modules. The 5-member family can be further classified into two subgroups (Figure 6).

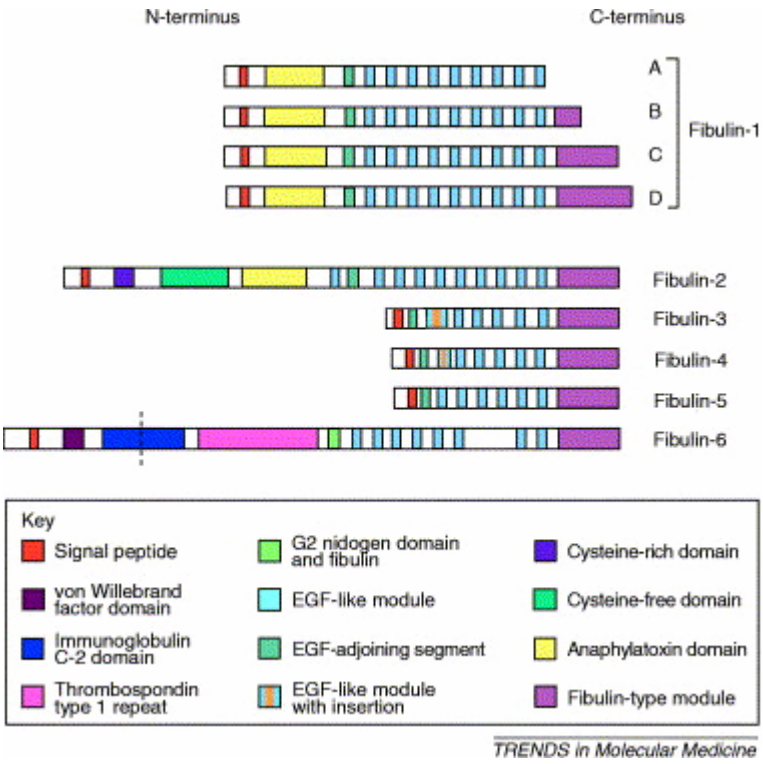


Figure 7: Structural features of the fibulin family. The fibulin family currently has six members, which possess a modular structure and have orthologues in species as divergent as humans and the nematode worm *Caenorhabditis elegans*. Domain information refers to the human forms of the fibulin family only. The four splice variants of fibulin-1, designated -1A to -1D, are shown. The large immunoglobulin domain of fibulin-6 is contracted for ease of visualisation, as indicated by the use of a dashed line (Gallagher et al., 2005).

Fibulin-1 and fibulin-2, the first subgroup, are substantially larger than the other three members of the family owing to the presence of an extra domain with three anaphylatoxin modules and higher numbers of tandem calcium binding and epidermal growth factor modules.Fibulin-1 with a molecular weight of 90000 kDa has variable C-terminal domain, while fibulin-2 at 200 kDa is the largest of all the fibulins since it possesses an additional N-terminal domain of approximately 400 aminoacids. Members of the second groups: fibulin-3, fibulin-4 and fibulin-5 are similarly small in size (50-60 kDa) and highly homologous to one another in the modular structure. They consist of a modified tandem calcium binding and epidermal growth factor domain at the N-terminus followed by 5 tandem modules and the fibulin C-terminal domain. Fibulin-1 and fibulin-2 are able to bind fibronectin, proteoglycans, tropoelastin and other basement membrane proteins, participating in extracellular supramolecular structures (Kobayashi et al., 2007).

2) Basement membrane assembly

The most interesting feature of the BM is the capacity of its components to self assemble and to form sheet-like structure. Cells at first assemble BM components into functional units (Type IV of collagen protomer, laminin trimers, nidogen/entactan and perlecan) inside the cell, and then they secrete them. Laminin polymerization is believed to initiate the BM scaffold formation at the basolateral surface cells. It is anchored to the cell by receptor proteins such as integrins or dystroglycans. Deposition of this polymer leads to association with type IV of collagen network. Nidogen/entactin bridges the laminin polymer and type IV of collagen network. The other components of the BM interact with the laminin polymer and type IV of collagen network to organize a functional BM on the basolateral aspect of the cell (Kalluri et al., 2003).

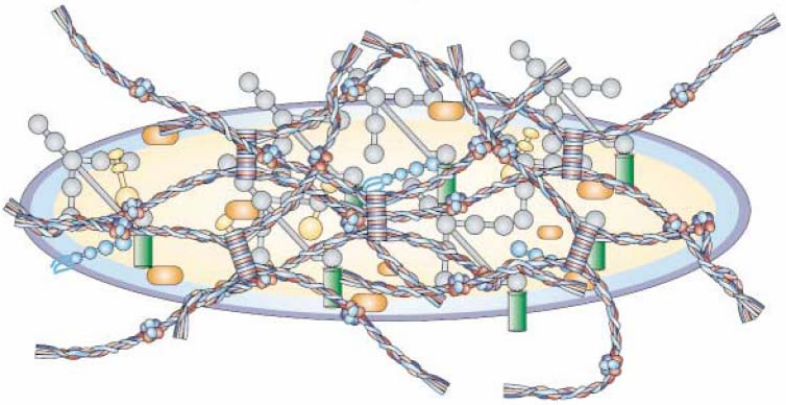


Figure 8: Basement membrane formatio. Cells first assemble BM components into functional units inside the cell (type IV of collagen protomers, laminin trimers, nidogen/entactin complex) and then secrete them. Nidogen/entactin bridges the laminin polymer and type IV of collagen network. The other components of the BM interact with the laminin polymer and the type IV of collagen network to organize a functional BM on the basolateral aspect of the cells

3) Angiogenesis and vascular basement membrane

Angiogenesis is an important biological process considered fundamental to reproduction, development and repair. In the adult, repair and reproductive angiogenesis occurs mainly as brief bursts of capillary blood-vessel growth that usually last only days or weeks. This physiological angiogenesis is tightly regulated by a variety of circulating or sequestred inhibitors that suppress proliferation of vascular endothelium.

The vascular basement membrane (VBM) is composed predominantly of type IV of collagen, laminin, enactin/nidogen and proteoglycans. This complex structure plays a pivotal role in angiogenesis. Infact most of this component sustain the growth and health of vascular endothelium, cryptic domains within these proteins also posses anty angiogenic activity. VBM organization is dependent on the assembly of a type IV of collagen network, wich is believed to occur via the C-terminal globular non collagenous domain (NC1)of collagen IV. Inhibitors of collagen metabolism have anti-angiogenic properties, supporting the notion that basement membrane collagen synthesis and deposition are crucial for blood vessels formation and survival (Maragoudakis et al., 1993, 1995) Figure 9.

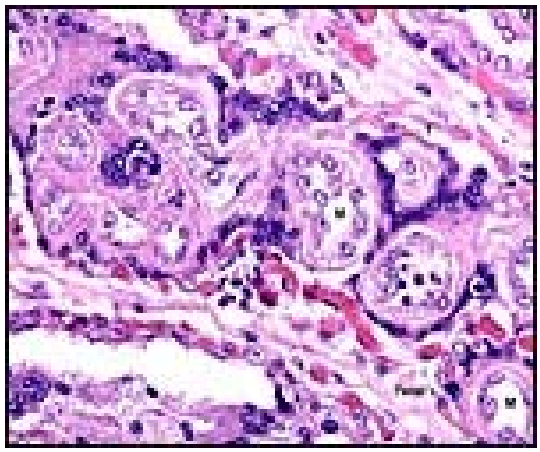


Figure 9: Overview of the vascular basement membrane structure and vessels formation..

Blood vessels are made by BMs, pericytes and vascular endothelial cells. During angiogenesis, vascular endothelial cells can proliferate as rapidly as bone-marrow cells. This increase on proliferation is one of the main events that is required for the formation of a new capillary blood vessel. Vascular endothelial cells proliferation is associated to the degradation of the VBM, which leads to sprouting of pre-existing microvessels. These vessels invade the ECM, form tubes and the tips of these tubes eventually connect each other to create loops that are capable of conducting blood flow (Kalluri, 2003). During angiogenesis endothelial cells are dislodged from existing blood vessels, then surrounded by provisional matrix, and eventually come to rest on capillary BMs that are composed of several matrix molecules. The angiogenic response is induced by growth factors such as vascular endothelial growth factor (VEGF), basic FGF (βFGF), platelet-derived growth factor (PDGF), chemokines and others. First, the capillary BM is degraded by several matrix-degrading enzymes such as MMPs , which are produced by stromal cells endothelial cells or by tumour cells themselves. During tumour angiogenesis these factors can also be produced by the immune cells that accumulate around the neoplastic cells even before angiogenesis is initiated. This vascular BM degradation serves multiple purposes, which include the liberation of endothelial cells to migrate and proliferate from their cell-surface integrins, the liberation of sequestred growth factors (VEGF, βFGF) and the detachment of the pericytes that surround and support the blood vessels. The detachment of endothelial cells are now in direct contact with interstitial provisional matrix components, such as vitronectin, fibronectin, type I collagen an thrombin. Pro-angiogenic factors induce endothelial cells to produce many of this matrix molecules. Therefore, provisional matrix provides proliferative cues to endothelial cells, whereas the assembled BM matrix provides growth-arresting cues Figure 10.

Figure 10: The angiogenic switch. Endothelial cells break free from their basement membrane and surrounding extracellular matrix, migrate, proliferate, and remodel, thus generating new blood vessels or "sprouts" from the parent vessel

4) Regulation of angiogenesis by MMPs

Progression of cancer is dependent on neoangiogenesis. Tumour progression is likely governed by relative levels of pro- and antiangiogenic factors: "the angiogenic balance". Influenced by oncogenes and tumor suppressor genes, disruption of the "angiogenic check point "can represent an important lethal step in the progression of cancer (Hamano et al., 2003) via the increase in angiogenic factors (such as VEGF) or decrease in the physiological levels of endogenous inhibitors of angiogenesis like tumstatin.

Recent studies have shown that MMP-9 and MMP-2 are required for the mobilization of the sequestred VEGF and the triggering of tumour angiogenesis (Bergers, et al., 2000).

Gelatinases (MMP-9 and MMP-2) degrade type IV of collagen , probably distrupting the organization of BM, leading to the release of BM-bound VEGF. (Figure 7). MMPs are predominantly produced by stromal and immune cells. After the matrix is degraded and VEGF is released, angiogenesis is initiated and tumours begin to grow and recruit more immune cells, fibroblasts and other stromal cells, which also produce VEGF and βFGF (Coussens et al., 2001).

MMP-9-mediated release of bound VEGF is potentially important in the very early stages of local tumour progression that are associated with the angiogenic switch.

As the BM undergoes MMP-mediated degradation and structural changes, cryptic domains of partially degraded collagens become exposed (Xu et al., 2001;Xu et al., 2005). These domains have been shown to provide important proangiogenic cues that were sequestred when the BM was fully assembled. Similarly, MMP-mediated degradation of the BM also leads to the generation of fragments with antiangiogenic activity, such as endostatin, arrestin, canstatin, tumstatin and other collagen fragments (Egeblad et al., 2002;Lee et al., 2002; Petitclerc et al., 2000).

Therefore while whereas the initial burst of MMP production releases BM-bound VEGF and other factors that initiate tumour angiogenesis, this need for a VEGF source leads as soluble VEGF is produced in large amounts by the infiltrating immune cells and by the increasing number of cancer cells themselves. As the tumour grows and more MMPs are produced, most of the BMs are degraded within the

tumour microenviroment and potentially elsewhere. As the BM degradation reaches completion, much of what remains are potential MMP-resistant products, such as endostatin, arrestin, canstatin and tumstatin, all angiogenic inhibitors. MMP-mediated degradation of BM can therefore act as both a positive (early stage) and a negative (middle to late stage) regulator of tumour angiogenesis (Kalluri, 2003; Pozzi et al., 2000; Pozzi et al., 2002) (Figure 11).

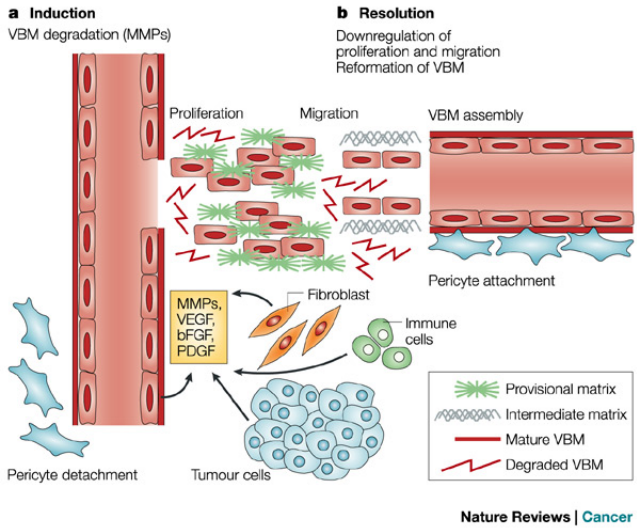


Figure 11: degradation of the vascular basement membrane by MMPs.
a) in response to growth factors and matrix metalloproteinases the VBM undergoes degradative and structural changes. The transition from mature and provisional VBM promotes the proliferation and migration of vascular endothelial cells. Growth factors, such as the endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF) are relased by BM and produced by tumor cells and immune cells. b) Together with the vascular endothelial cells and pericytes, the VBM mediates formation of a new blood vessel.

II) Matrix Metalloproteinases (Matrixin): Structure and Function

1) The Metzincin superfamily

Matrix metalloproteinases belong to a family of zinc and calcium dependent endopeptidases called Metzincin. A quantitative comparison of the three-dimensional structures of the matrixins (MMPs) and the other three protein families (i.e., the astacin, the serralysins, the adamalysins) has revealed striking topological similarities between the catalytic modules of all these proteins. This superfamily is characterized by a catalytically active zinc ion on the active site and is distinguished by a highly conserved motif containing a consensus sequence, HEXXHXXGXXH (single letter code; X is any amino acid residue), which is involved in metal ligation. The three histidines of the conserved consensus sequences serve as ligands for the zinc, the glycine helps the formation of th loop around the metal and the glutamic acid is believed to transfer hydrogen atoms and polarize a zinc-bound water molecule for nucleophilic attack on the scissile peptidic bond of substrate (Stocker, 1995).

2) Structural complexity of MMPs

To date the MMP gene family in humans encodes 25 homologous proteinases (MMPs 1-3, 7-28) and three pseudo-genes. Like many extracellular proteins, MMPs are multi-domain proteins that share similar primary, secondary and tertiary structures.
All MMPs possess a leader domain involved in enzyme secretion, a pro-domain that is auto-inhibitory; and a catalytic domain required for enzyme activity. Most of MMP members possess also a C-terminal hemopexin-domain which is involved in substrate recognition.
The domain composition is different for each MMP and it is listed in table I. Functionally, the most important is obviously the catalytic domain. The prodomain, which is responsible for the enzyme latency, extends from the catalytic domain to the N-terminus of the enzyme. At the C-terminus of the catalytic domain a length-varying linker connects to hemopexin C-terminal domain which is present in most MMPs except for MMP-7 and MMP-26 (Overall et al., 2002).

The structure of domain motifs are illustrated in Figure 11A: prodomain (Figure 11B), catalytic domain (Figure 11C), fibronectin-like domain (Figure 11D), hinge region, hemopexin -like domain (Figure 11E).

The MMPs can be divided into 5 main groups according to their domain composition and their ability to degrade individual component of extracellular matrix:

- Matrilysins (MMP-7 and MMP-26) are MMPs that lack the hemopexin C domain;
- Collagenases (MMP-1, MMP-8 and MMP-13) are composed of a catalytic domain and hemopexin -like domain;
- Stromelysins (MMP-3, MMP-10 and MMP-11) possess the same domains composition of collagenase class;
- Gelatinases (MMP-2 and MMP-9) have within the catalytic domain a compact collagen binding domain called fibronectin-like domain;
- Membrane Type MMPs (MT-MMP) (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24 and MMP-25) are inserted in the plasma membrane by a trasmembrane segment or a glycosylphosphatidylisnositol (GPI).

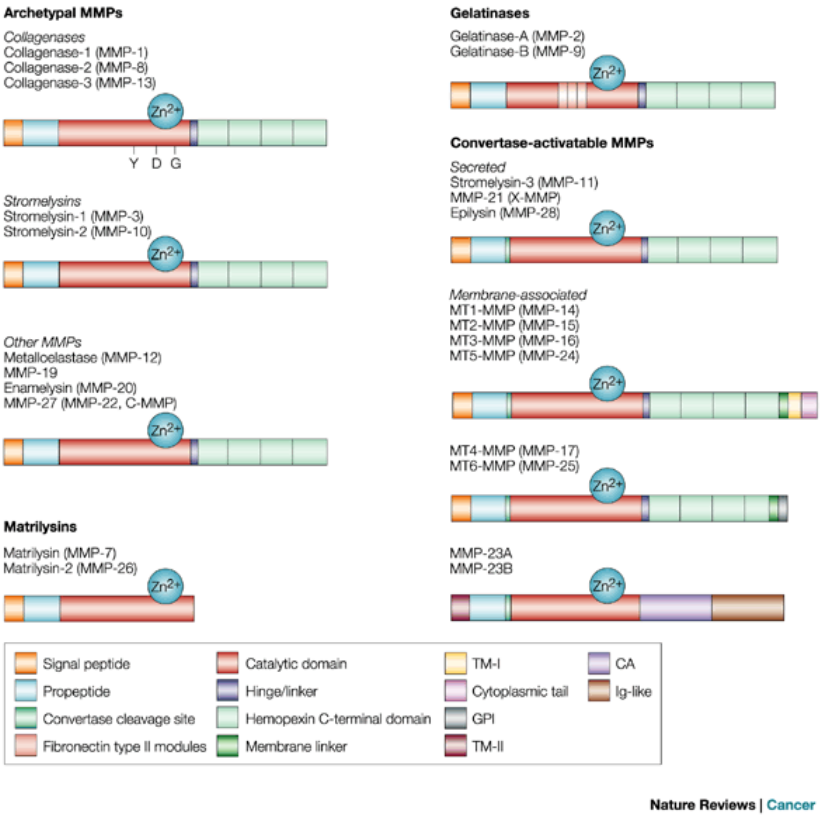


Table I. Schematic representation of the structure of the 24 human matrix metalloproteinases (MMPs), which are classified into four different groups on the basis of domain organization. Archetypal MMPs contain a signal peptide (necessary for secretion), propeptide, a catalytic domain that binds zinc (Zn^{2+}) and a hemopexin carboxy (C)-terminal domain. Y, D, and G represent tyrosine, aspartic acid and glycine amino acids that are present in the catalytic domain of all collagenases. Matrilysins contain the minimal domain organization that is required for secretion, latency and catalytic activity. Gelatinases contain fibronectin type II modules that improve collagen and gelatin degradation efficiency. Convertase-activatable MMPs contain a basic insert in the propeptide that is targeted by furin-like proteases (convertase cleavage site). MMPs that belong to this group can be secreted enzymes, or membrane-anchored via GPI (glycosylphosphatidylinositol), type I or type II transmembrane (TM) segments. MMP-23A and MMP-23B contain unique cysteine array (CA) and immunoglobulin (Ig)-like domains in their C-terminal region (Overall et al., 2002).

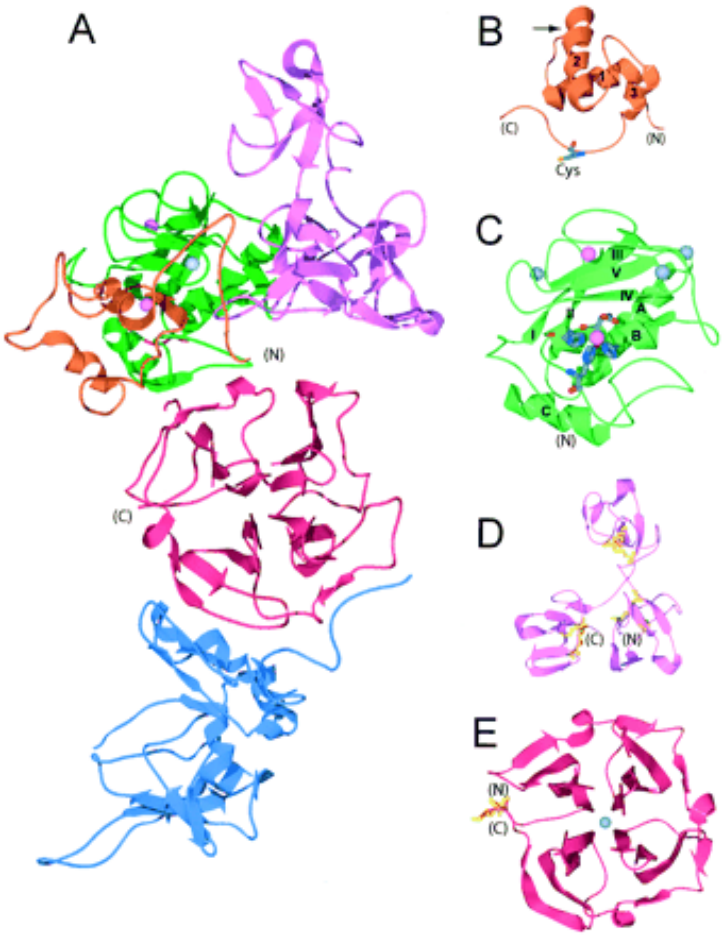


Figure 11. 3D structure of MMPs: ribbon diagram of MMP structures. A) proMMP-2-TIMP2 complex (1GXD) is shown. Orange indicates propeptide; green, catalytic domain; pink, fibronectin-like domains; red, hemopexin -like domain; and blue TIMP-2. Zinc atoms are pink, and calcium are gray. B) In the MMP-2 propeptide, the cysteine swtch motif is shown. The harrow indicates the position of the initial cleavage resulting in partial activation. C) The catalytic domain of MMP1 is shown. The beta strands are numbered I trough V; the alpha helices are labelled A through C. The N-terminal (N) to C-terminal (C) ordered of the beta strands and alpha helices is I-A-II-III-IV-V-B-C. The histidines coordinating the active site zinc and the active-site glutamic acid are shown. D) The 3 fibronectin-like domains of MMP-2 are shown with their 2 disulfide bonds each. E) The hemopexin -like domain of MMP-1 with 4 beta propeller blades is shown. A disulfide bond is seen between blades I and IV (Visse et al., 2003).

Many of the MMPs are specifically regulated at the level of gene expression, but their production as inactive proenzymes is another important level of regulation (Sternlicht et al., 2001).
All matrixins are synthesized as pre-pro-enzymes and secreted as inactive pro-MMPs in most cases. The pro-peptide domain (about 80 amino acids) has highly conserved unique PRCG(V/N)PD sequence. The Cys within this sequence ('the cysteine switch') coocrdinates the catalytic zinc to maintain the latency of pro-MMPs (Massova et al., 1998).
Structure of the pro-domain is known for MMP-2, MMP-3 and MMP-9; it consists of its three α-helices and connecting loops (Figure 2B). The first loop between helix-1 and helix-2 is a protease-sensitive 'bait region'. An extended peptide region after helix lies in the substrate binding cleft of the catalytic domain. As already mentioned, this region contains the conserved cysteine switch, which forms a fourth ligand of active site, via its side chain thiol group, keeping the zymogen inactive.
This sequence is missing in stromelysin (MMP-11) and MT1-MMP (MMP-14) which were shown to be activated intracellulary by furin, while MMP-23 has a proprotein processing sequence RX(K/R)R at the C-terminal end of the propeptide (Nagase et al., 1999).
It is notable that the orientation of the propeptide backbone is opposite to that one of peptide substrate since it interacts with the active site cleft. However, the hydrogen bonds that it makes with the active site are identical to those of substrate backbone (Visse et al., 2003).

a) The Catalytic Domain and Active Site

The catalytic domains of MMPs are generally very similar with sequence similarities in the range of 50-88% and identities in the range of 33-79 % (Terp et al., 2002). The common structural features includes three α-helix (A, B and C) and a β-sheet consisting of four parallel and one antiparallel strand and connective loops with two zinc ions and between one and three calcium ions.
Their catalytic domains all exhibit an overall spherical shape which can be divided into two subdomains by the substrate-binding cleft with the zinc atom at its bottom. The N-terminal half of the catalytic domain is made up from a twisted β-sheet covering two long α-helices; the central α-helix in the

active site contains the two histidine residues of the consensus motif HEXXHXXGXH (Stocker et al., 1995).
The third histidine zinc ligand of the metzincins is part of the C-terminal half of the catalytic domain being positioned three residues downstream of the conserved glycine. Generally, this C-terminal portion exhibits a long α-helix which packs against the surface of N-terminal domain. The polypeptide chain connecting the third histidine to the C-terminal helix forms a loop made possible by the conserved glycine which interrupse the α-helics, that ends at the catalytic zinc in a unique, tight 1.4 'Met turn' (Bode et al., 1995) (Figure 12)**.**
The very N-terminal region runs parallel to helix C before intruding into the molecular body at a strictly conserved tryptophan residue; furthermore the conformation of the N-terminal portions (consequences of pro enzyme activation) is of extreme importance for the regulation of their proteolytic activity. In fact, two structures of the MMP-8 catalytic domain resulting from the cleavage at two different sites, leaving either Met-80 or Phe-79 as the N-terminal residue display different structures (Reinemer et al., 1994). The latter form is 'superactivated', as Phe-79 forms a salt bridge with a Asp-232 thereby preventing the N-terminal sequence from interference with active site. The result is a 3 fold increase in activity compared with activation cleavage at Met 80 (Knauper et al., 1993; Gioia et al., 2002).

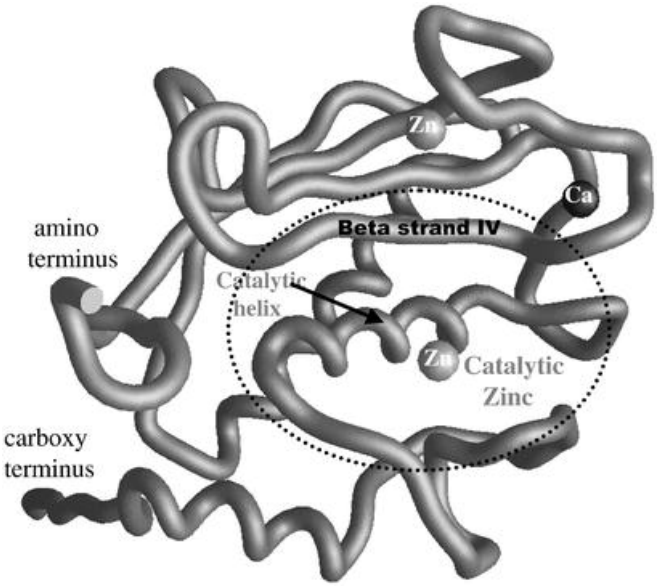


Figure 12.The catalytic domain of ligand-free collagenase MMP-1.
***Dark spheres* calcium atoms*; light spheres* zinc atoms (Borkakoti et al., 2000).**

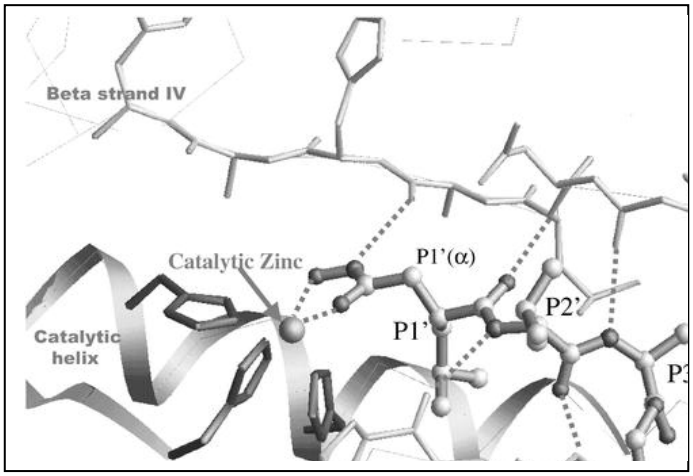


Figure 13.The typical mode of binding of potent inhibitors to matrix metalloproteinases.
The figures illustrates the interaction of the peptidic inhibitor (Ro 32-4724, ball and stick representation) at the active site of MMP-1 (area identified in figure 3) (Borkakoti et al., 2000).

Catalysis mechanism

As described above, the catalytic zinc is coordinated by the three histidine residues; in the absence of a substrate or inhibitor, a water molecule forms an additional fourth ligand to zinc and is entrapped between the catalytic glutamic acid of the HEXXHXXGXXH motif and the metal. In all MMPs the carbonyl group of scissile peptide bond points towards the catalytic zinc and therefore can be polarized (Figure 13).
The peptide hydrolysis is assisted also by the carboxyl group of the catalytic glutamate, in fact the catalytic water molecule intercalates between this carbonyl group and the glutamate, thereby facilitating the nucleophilic attack of the water molecule on the carboxyl carbon of the peptide scissile bond, and giving rise to a pentacoordinate transition state (Stocker et al.,1995) (see Figure 14).
The tetrahedral intermediate is presumably stabilized by both the zinc and a carbonyl group of the first alanine residue of the edge strand sIV. Simultaneously, one water proton could be transferred to the amino group via the glutamic carboxylate (acting as a proton shuttle); after the cleavage of the peptide bond and the transfer of a second proton this amino group could leave the enzyme-substrate complex (together with the N-terminal substrate fragment) (Bode et al., 1994). In addition, proton transfer to the peptidic nitrogen possibly facilitated by the catalytic glutamate, completes the catalytic reaction, enabling the release of cleavage products and of the free enzyme (Stocker et al., 1995).

Substrate specificity pockets

Although all MMP structures reported to date possess similar core domains, important differences in the side-chains and surface loop alter the size, shape and chemical composition of the specificity subsites.
The substrate-binding domain cleft is formed by strand IV and helix B (see Figure 13). The active site is a cavity spanning the entire enzyme and it has been shown that a substrate containing at least six amino acids (three on each side of the scissile bond) is required for the proteolytic activity of MMPs: these six amino acids occupy the subsites S3-S3'(notation according to Schechter and Berger) (Terp, et al.2000) (Figure 14).
Closer examination of the structures of catalytic domains revealed that a conserved aspartic acid is found in the vicinity of the methionine turn, the

side chain of which is buried inside the core of the domain (Massova, et al. 1998). The substrate binds into the catalytic site cleft from the left to the right with respect to its N- and C-termini, and the carboxyl group of the peptide bond coordinates with the active site zinc. A pocket to the right of the active site zinc, called S1' specificity pocket, accommodates the side chain of the substrate residue, which becomes the new N-terminus after cleavage. The size of S1' pocket varies among the MMPs and this is one of the major determining factors of substrate specificity (Visse, et al.2003).

Substrate specificity among MMPs is achieved mostly on the recognition pocket S1', that is of different length and amino acid composition in the individual MMP. The S1' pockets, which are surrounded by a loop, are generally quite large in all MMPs. However, in the X-ray structure of MMP-1 an arginine defines the bottom of the pocket, whereas in MMP-7 a tyrosine fulfills this purpose, leading to a restriction of the pocket (Terp et al., 2002 and Yaun et al, 1999). In addition, it has been reported that flexibility of this site in some cases could lead to an induced-fit process upon ligand binding; in fact, in MMP-1 the Arginine movements make the pocket able to accommodate larger substituents (Lovejoy et al., 1999). In all other MMPs, this residue is either a leucine or a threonine and the pocket adopts an extended shape. This includes MMP-8, although it resembles MMP-1 by having an arginine defining the bottom of the S1' pocket (Terp et al., 2002).

If we compare the catalytic domain of MMP-2 with that of MMP-8 prominent differences emerge on S1' loop: in particular, the MMP-2 sequence (YTYTKN--FRL) is two residues shorter than that of MMP-8 and there are seven substitutions including five non-conservative changes (YAFRETSNYSL) (Feng et al., 2002).

Traditionally, S1' subsite has been labelled as the specificity pocket because of its significant size and shape differences among the rigid x-ray structures of various MMPs. However, modification of P1' part of the ligands led to both increased selectivity and falures that were explained by observed changes in the structure of the pocket upon inhibitor binding.

The S2' and S3' subsites are partly solvent exposed and can accommodate a wide range of functionalities, while differences in the residues surrounding these sites might be used for selectivity purposes.

The primed sites of active site have been described in detail, whereas the unprimed sites have not been examined deeply. However, the importance of other parts of binding site, especially the unprimed side, for the design of more potent and selective inhibitors has been demonstrated as well (Lukacova et al., 2004).

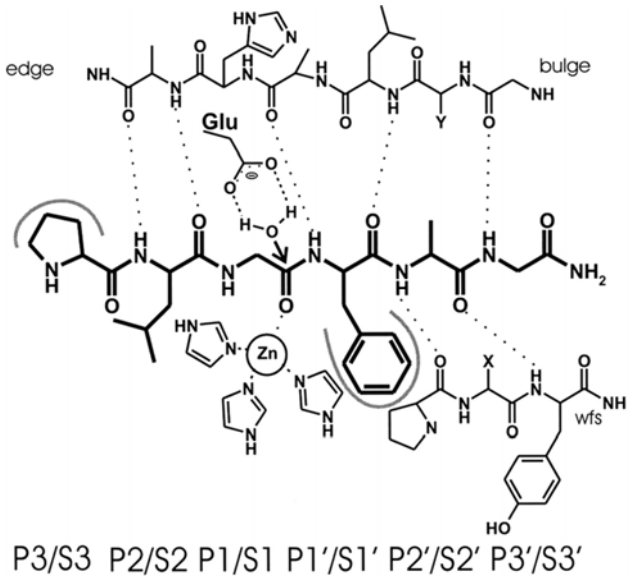


Figure 14. Peptide substrate and inhibitor interaction and specificity. Schematic drawing of the putative encounter complex between a Pro-Leu-Gly-Leu-Ala-Gly-amide hexapeptide substrate and the MMP active site. The substrate polypeptide chain (boldconnections) lies antiparallel to the bulge-edge strand (top) and parallel to the S1' wall-forming segment (bottom) forming up to five and two, respectively, intermain-chain hydrogen bonds (dashed lines) (Bode et al., 1999).

b) *The hemopexin -like domain*

The C-terminal domain of the MMPs, which is present in all members except the matrilysins, shows strong sequence similarity to members of the hemopexin family, including hemopexin, α-subunit of integrins and vitronectin (Smith et al., 1999). The common functional property possessed by these hemopexin-containing proteins seems to be the participation in quite specific protein-protein and protein-ligand interaction (which differ among various MMPs). All hemopexin-like domains have a β-propeller topology with pseudo four-fold symmetry, with sequence homology to a haem-binding and transporting protein from serum (Bode et al., 1995). The typical hemopexin-like domain comprises ~200 amino acid residues and shows a fourfold internal sequence repeats: the four blades of propeller are made up of antiparallel four β sheets arranged around a tunnel harbouring a number of ions (calcium and chloride). The first and the fourth blades are linked by a disulphide bond which is conserved across all the MMPs (Murphy et al., 1997) (Figure 11E). When present, the hemopexin-like domain also influences TIMP (tissue inhibitor metalloproteinase) binding, the binding of certain substrates, membrane activation and some proteolytic activities (Sternlicht et al., 2001).

c) *The hinge region*

The N- and C-domains of the MMPs seem to be packed as separate entities in the crystal with connecting flexible linker peptide or 'hinge' which is rather loosely arranged. The hinge is composed of 2-72 residues (Lauer-Fields et al., 2002), being short (16 residues) in the collagenases where displays a motif resembling those observed in each α-chain of triple helical collagen (polyproline II) (Knauper et al., 1997). The polyproline II conformation of collagen chains possess backbone ϕ-γ angles similar to antiparallel and parallel β-sheets (Perona et al., 1997). The residues of the hinge region also influence substrate specificity; the capacity to cleave triple-helical collagen obviously depends on the correct interplay between the catalytic and the hemopexin-like domain. This might be co-determined by

the length and the surface-guiding properties of the linker peptide (Bode et al., 1995).

d) *The fibronectin-like domain*

The gelatinases (i.e, MMP-2 and MMP-9) have an additional domain consisting of three tandem copies of 58 amino acid residues forming the fibronectin type II like module, which are inserted between the fifth β-strand and the catalytic helix (adjacent and aminoterminal to S3' subsite). The structure of each fibronectin-like domain consists of two antiparallel β sheet, connected with a short α-helix and stabilized by two disulfide bonds (Visse et al., 2003). A portion of this domain is called collagen binding domain (CBD), since it appears to be important for binding gelatin and collagen (Lauer-Fields et al., 2002).

3) Activation of proMMPs

a) *Intracellular activation*

Most proMMPs are secreted from cells and activated extracellulary. Pei, in 1995, first demonstrated that proMMP-11 is activated intracellulary by furin. ProMMP-11 possesses a furin recognition sequence, KX(R/K)R, at the C-terminal end of the propeptide. Several others MMPs, including the six MT-MMPs, MMP-23 and MMP-28, have a similar basic motif in the propeptide. All other MMPs lack a furin-susceptible insert and are thus activated outside the cell following their secretion.

b) *Stepwise activation mechanism*

MMPs can be activated by proteinases or in vitro by chemical agents such as thiol-modifying agents (4-aminophenylmercuric acetate, $HgCl_2$ and N-ethylmaleimide), oxidized glutathione, SDS, chaotropic agents and reactive oxygens (Figure 15) (Visse et al., 2003).
Low pH and heat treatment can also lead to activation. These agents most likely work through the disturbance of cysteine-zinc interaction of the cysteine switch. Studies of proMMP-3 activation with a mercurial compound have indicated that the initial cleavage occurs within the propeptide and this

reaction is intramolecular rather than intermolecular. The subsequent removal of the rest of the propeptide is due to intermolecular reaction of generated intermediates. In vivo, it has been shown that NO (nitrix oxide) activates proMMP-9 by interacting with the thiol group of cysteine switch and forming an S-nitrosylated derivative (Visse et al., 2003).
Proteolytic activation of MMPs is stepwise in many cases. The initial proteolytic attack occurs at an exposed loop region between the first and the second helics of propeptide. The cleavage specificity of the bait region is dictated by the sequence found in each MMP. Once a part of the propeptide is removed, this probably destabilizes the rest of the propeptide, including the cysteine switch-zinc interaction, which allows the intermolecular processing by partially activated MMP intermediates or other active MMPs. Thus, the final step in the activation is conducted by a MMP.
The stepwise activation system may have evolved to accommodate finer regulatory mechanisms to control destructive enzymes, in as much as TIMPs (tissue inhibitor of metalloproteinases) may interfere with activation by interacting with the intermediate MMP before it is fully activated.

c) *Cell surface activation of proMMP2*

The extracellular activation of most MMPs can be initiated by other already activated MMPs or by several serine proteinases that can cleave peptide bonds within MMP prodomains. However, proMMP-2 is refractory to action of serine proteinases and it is instead activated at the cell surface through a unique multistep pathway involving MT-MMPs (Figure 16).
According to the present knowledge, a cell surface MT1-MMP binds the N-terminal domain of TIMP-2 being inhibited; the C-terminal domain of the bound TIMP-2 acts as receptor for the hemopexin-like domain of proMMP-2. Then, an adjacent, MT1-MMP cleaves and activates tethered proMMP-2. Following the initial cleavage of proMMP-2 by MT1-MMP, a residual portion of MMP-2 propeptide is removed by another MMP-2 molecule to yield a fully active, mature form of MMP-2 (Sternlicht et al., 2001).

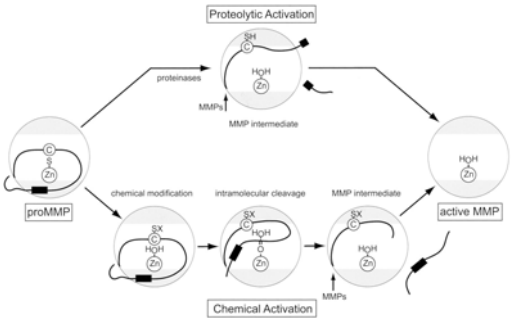


Figure 15. Stepwise activation of proMMPs. ProMMPs secreted as inactive zymogens can be activated by protease (top pathway) or by non proteolytic agents (bottom pathway). The catalytic domain is represented as a graycircle, with the active site cleft shown in with (not in scale), containing the catalytic zin (Zn). The propeptide is schematically shoen as a black line containing the bait region (black rectangle) and the cysteine switch (C). SH indicates the sulfhydryl of the cysteine. Activation by protease is mediated by cleavage of the bait region; this partly activates the MMP. Full activations achieved by completed removal of the propeptide by intermolecular processing. Chemical activation relies on modification of the cysteine switch sulfhydryl (SX), resulting in partial activation of the MMP and intramolecular cleavage of the propeptide. Full activity results from the removal of the remainder of the propeptide by intermolecular processing (Visse et al., 2002).

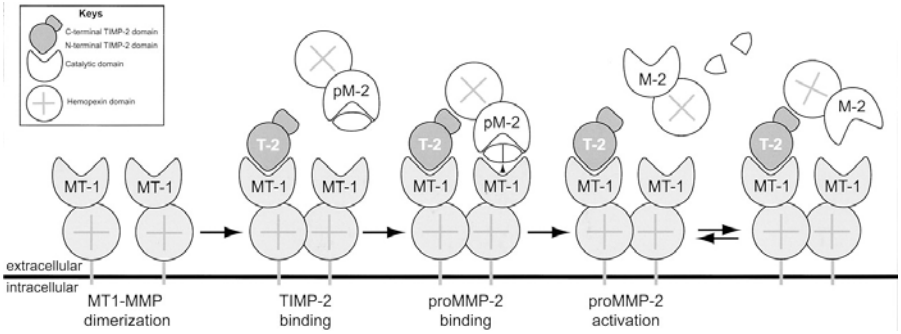


Figure 16. Model of proMMP-2 activation by MT1-MMP and TIMP-2. active MT1-MMP (MT-1) on the membrane binds a molecule of TIMP-2 through its hemopexin-like domain. The second, active, MT1-MMP then cleaves the region of proMMP-2, thereby partly activating it. The MMP-2 (M-2) dissociates from the membrane and is fully activated by intermolecular processing (Visse et al., 2002).

4) Inhibition of MMP activity

Beyond their classical connective-tissue-remodelling functions, MMPs are known to precisely regulate the function of bioactive molecules by proteolytic processing. Under physiological conditions, MMP activity is controlled at least at three levels:

- transcription,
- proteolytic activation of zymogen form,
- inhibition of the active enzyme by endogenous inhibitors.

Most MMPs are expressed in adult tissues at low levels or not at all in resting condition. However, several cytokines and growth factors as well as physical cellular interactions provide stimuli that can rapidly induce MMP expression (Stamenkovic et al., 2003). Thus, cell use various strategies to regulate extracellular proteases; in this paragraph, the role of metalloproteinase inhibitors is reviewed (Figure 8 Baker et al., 2002).
A greater understanding of regulatory mechanism that controls MMP activity provides several new avenues for therapeutic intervention, since many drugs are designed to target these key regulatory points (Figure 17).

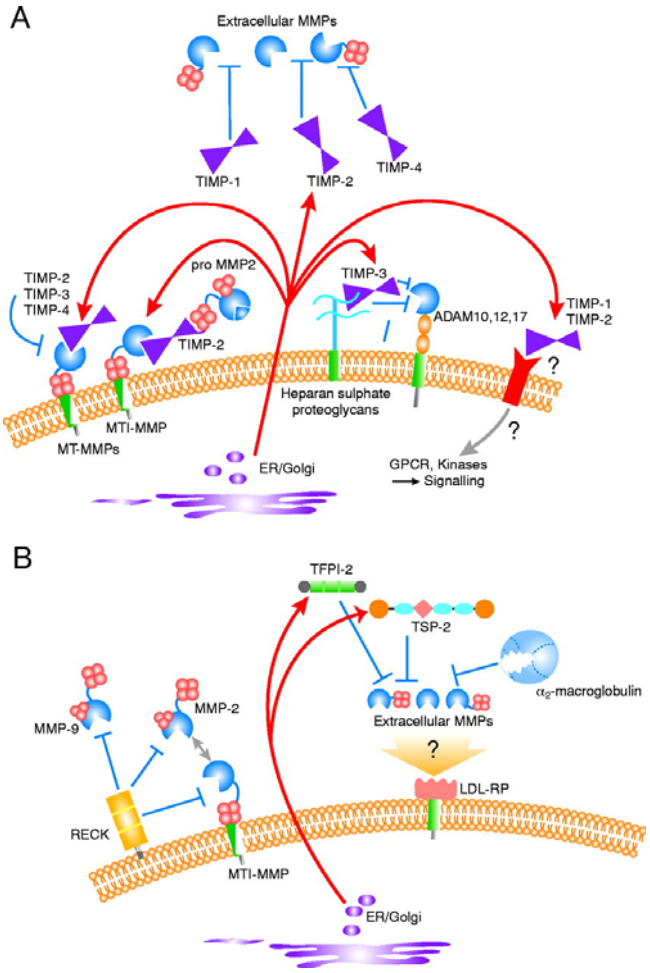


Figure17. Metalloprotease inhibitors in the pericellular enviroment. A) tissue inhibitors of matrix metalloprotase (TIMPs). TIMPs- 1-4 are largely MMP inhibitors modulating the activity of soluble, matrix bound and cell associated MMPs. TIMP- 3 is an extracellular matrix protein, probably bound to heparan sulphate proteoglycans and it is a potential inhibitor of the function of some membrane associated ADAMs. TIMP2 acts in junction with MT1-MMP as receptor for the proform of MMP-2 at the cell surface. Allowing an efficient activation and focussing of the active form of this soluble protease. In the same cell types, TIMP-1 and TIMP2 may have receptors directly linked to intracellular signalling pathways regulating cell behaviour. B) other inhibitors. RECK (Reversion inducing cysteine rich protein with Kazal motif) is a GPI-anchored glycoprotein that binds and inhibits a number of MMPs. The pan protease inhibitor alpha 2 macroblobulin, although very large, has some access to the pericellular space in vascularised tissue and may be involved in MMP endocytosis through the low density lipoprotein (LDL-RP). The roles of LDL-RP in MMP-2 removal via a thrombospondin-2 (TFPI-2) has also been described as an MMP binding agents (Baker et al., 2002).

a) *Endogenous inhibitors*

MMP activity is tightly controlled by several endogenous inhibitors. In tissue fluids, the principal MMP inhibitors is $\alpha_2$macroglobulin, a large serum protein, which binds MMPs and creates a complex that is itself bound irreversibly (Yang et al., 2001; Woessner et al., 1998).
However, the most throughly studied MMP inhibitors, are TIMPs (Tissue Inhibitors Metallo Proteinases). Four human TIMPs have been characterized thus far; they are small molecules of 21-28 kDa which bind MMPs in a 1:1 stochiometric ratio and reversibly block MMP activity. TIMPs, which are anchored to the extracellular matrix or secreted extracellulary ,differ in their expression pattern.
Among other molecules capable of regulating MMP proteolytic activity we underline thrombospondin-2 and RECK (reversion-inducing cysteine-rich protein with Kazal domain motifs), a GPI-anchored glycoprotein that suppresses angiogenic sprouting (Stamenkovic et al., 2003). Other key regulators of extracellular matrix are TFPI2 (tissue-factor patway-inhibitor-2), a serine protease inhibitor that can act as a MMP inhibitor and PCPE (procollagen C-terminal proteinase enancer), a molecule which possesses a significant inhibitory activity. Other latent MMP inhibitors might be hidden in the NC1 domains of type IV collagen or in the laminin-binding domain of agrin, which are structurally similar to TIMPs (Overall et al., 2002) (Figure 17). However, it may be interesting to outline that endogenous inhibitors may also act as activators. This unexpected function has been quite well described for TIMP2 in the case of MMP2 activation.

b) *Exogenous MMP inhibition*

In recent years the MMPs have been implicated in a variety of important diseases including cancer (growth and metastasis), atherosclerosis, osteo- and rheumatoid arthritis and emphysema (Palvlaki et al., 2003). However, the evidence has been, consisting largely of correlations such as higher rates of metastasis accompanied by higher level of MMPs. Recent works, based on the development of knock-out mice for several MMPs, suggest more strongly that there is a rational point in developing inhibitors to MMPs with the goal of reversing disease processes. There are several approaches to inhibit MMP

gene transcription based on targeting extracellular factors, signal-transduction pathways or nuclear factors that activate expression of these genes. In addition, ribozymes or antisense constructs down-regulate MMP production by targeting MMP transcripts (Overall et al., 2002).

Inhibition of active MMPs by chelators

Several mechanisms reduced the tissue levels of MMPs; a great deal of attention has been given to chelating agents that bind zinc at the catalytic centre. Most attention has been focussed on improvements of hydroxamates which belong to chelators compound with no specificity and would block any enzyme containing zinc. The hydroxamate moiety offers two oxygen atoms zinc binding as well as nitrogen atom to the carboxyl group of the backbone of protein and results in compounds with nanomolar affinity (Borkakoti et al., 2004).
Several classes of MMP inhibitors have been discovered; the most common is containing a Zn-liganding hydroxamic acid, carboxylic acid or thiol group attached to a small peptide fragment capable of binding to specificity pockets of the MMP enzyme. Ligands coordinate the catalytic zinc atom and interact with either the S1-S3 or S1'-S3' subsites (Figure 14). The vast majority of inhibitors described to date bind in an extended conformation in the right side (S1'-S3') of the active site. Recently, the thiazole class of inhibitors was discovered and determined to interact with the left side (S1-S3) of the active site (Yuan et al., 1999).

Natural inhibitors

Unfortunately, most of synthetic compounds produced up to date are quite specific toward the rest of MMPs and at therapeutic doses are toxic even though less toxic than other anticancer agent. However, peptido-mimetic MMP inhibitor developments are yet in-phase-III studies in clinical trial (Pavlaki et al., 2003). On the other hand, among the biologically active components from natural products, green tea polyphenols caused the strong inhibition of MMPs. More particularly, it has been reported that natural extracts components (such as epigallo chatechin gallate EGCG) are able to inhibit the enzymatic activity of various MMPs as well as the activation of proMMP-2 (Demuele et al., 2000).

III) Gelatinases and degradation of type IV collagen

Endothelial cell invasion is an essential event during angiogenesis, a process that consists on new blood vessels formation (Nguyen et al., 2001). Angiogenesis has been identified as a casual or contributing factor in several pathologies, including cancer, where it is a rate-limiting step during tumour progression.

Matrix metalloproteinases (MMPs) are a family of soluble and membrane-anchored proteolytic enzymes that can degrade components of the extracellular matrix (ECM) as well as a growing number of modulators of cell function. Enhanced expression of MMP-2 and MMP-9 has been observed in different kind of cancers, such as breast, colon, lung, skin, ovary and prostate. In particular, gelatinases have been linked to angiogenesis. Potential roles for these proteases during the angiogenic process include degradation of the vascular basement membrane and perivascular ECM components, unmasking of cryptic biologically relevant sites in ECM components, modulation of angiogenic factors and production of endogenous angiogenic inhibitors (Handsley and Edwards, 2005). Gelatinases cleave the main component of the vascular basement membrane, type IV of collagen, leading to VEGF release, necessary to endothelial cells proliferation. (Handsley and Edwards, 2005).

Moreover, vascular basement membrane cleavage gives rise to the formation of fragments with anti angiogenic activity.

1) Type IV of collagen structure

Type IV of Collagen is the most abundant component of the basement membrane, where it is crucial for its stability and assembly. It is termed network-forming collagen to its capability to self assemble into network structure. This proprety makes it different from the fibrillar collagens (type I, II and III). Type IV of collagen is formed by the association of three α chains in a primary structure called protomer. Six different type IV of collagen α chains have been identified codified by six different genes: α1(IV), α2(IV), α3(IV), α4(IV), α5(IV) and α6(IV). The type IV collagen α-chains have similar domain structures and share between 50-70% homology at the amino-acid level. Each α-chain can be separated into three domains: an amino terminal 7S domain, a middle triple helical domain, and a carboxy-terminal globular non collagenous (NC)-1 domain (Figure 18). The

triple-helical part is the longest domain (about 1,400 amino acids (aa) in length), with 22 interruptions of the classical Gly-X-Y sequence motif that is characteristic of collagens. The NC1 domain of each α-chain is about 230 aa in length. α–chain Usually type IV of collagen of the vascular basement membrane is formed by two α1 chain and 1 α2 chain that coassemble to form a trimer called protomer given by the interaction of the C-terminal domain of each α-chain. Type IV of collagen formation begins with the protomer formation and proceeds with protomer trimerization like a zipper from the carboxy-terminal end, resulting in a fully assembled protomer. The assembled protomer is flexible and can bend at many triple-helical interruption points in the molecule. The next step in the assembly is the type IV collagen dimer formation. Two type IV collagen protomers associate via their carboxy-terminal NC1 trimers to form an NC1 hexamer. Next, four protomers interact at the glycosylated amino-terminal 7S region to form tetramers. These interactions form the nucleus for a type IV collagen scaffold. The scaffold evolves into a type IV collagen suprastructure, with the help of end to end assiciations and also lateral associations between type IV collagen protomers (Kalluri, 2003).

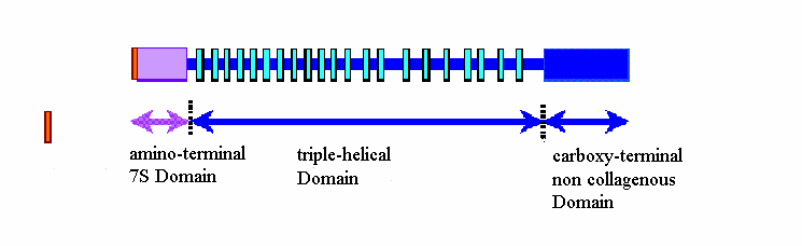


Figure 18: α-chain secondary structure. We can observe: at the N-terminal domain called 7S-domain formed by several sequences repeats; in the middle the triple helical domain or collagenous domain and at the C-terminal domain the so called non collagenous domain or NC1-domain (Kalluri, 2003).

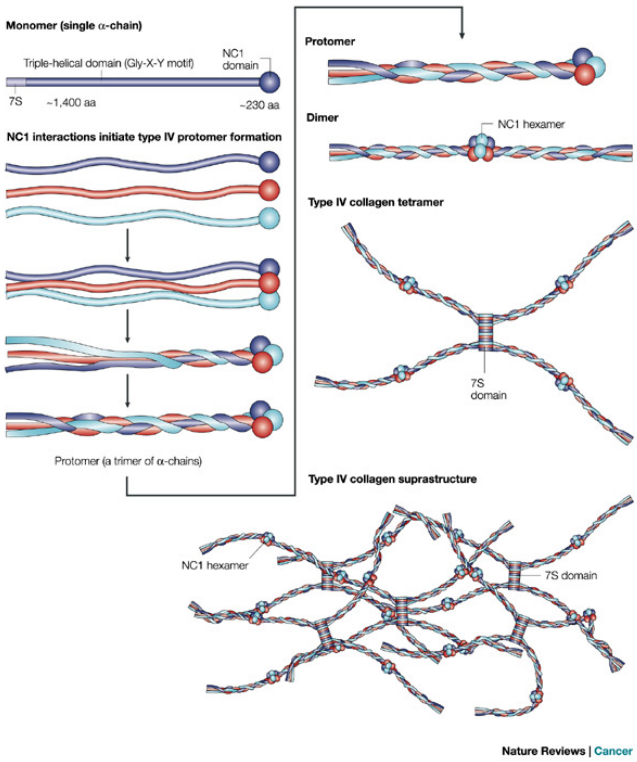


Figure 19: Type IV of collagen suprastructure formation. Type IV of collagen suprastructure begins with the formation of a primary structure called protomer, given by the interaction of the NC1 domain of three a-chains, and proceeds with dimer, hexamer and tetramer constitution to form finally the collagen IV complex structure (Kalluri, 2003).

2) Gelatinases cleavage site for degradation of type IV collagen

Gelatinases are a particular class of the extracellular degrading enzymes family called matrix metalloproteinase or MMPs. While MMPSs play a critical role in normal and pathological processes involved in the remodeling of the extracellular matix (ECM) gelatinases are involved in the remodeling of BM structures. The BM structure is generally formed by different kind of glycoproteins, such as collagen IV, that is the major component of the vascular basement membrane.In vitro studies have shown that triple helical collagen IV molecules can be hydrolyized by several members of MMP family including gelatinases . Of particular interest is that the contribution of the gelatinases to the degradation of collagen IV seems to be associated to tumour metastasis, a process known to involve alterations in VBM integrity. This is also confirmed by previous studies that have shown that the gelatinases are localized on the surface of tumour cells (Ginestra et al., 1997). The degradation of VBM by gelatinases has been postulated to take place at areas of cell-matrix contacts (Werb, 1997). Searching for potential pro-MMP-9 surface binding molecules, Fridman et al. identified a surface bound α2(IV) chain of collagen IV that specifically formed a high affinity complex with pro-MMP-9 and plays a role in targeting pro-MMP-9 at relevent areas of proteolysis. The proteolysis of the collagen IV network by MMP-2 and MMP-9 seems to bring about also to the formation of fragment with an anti proliferative activity. In particular, MMP-2 has the capability to liberate some cryptic domains from type IV of collagen with anti angiogenic activity . Infact, the non collagenous domain (NC1) of α1 (arrestin) and α2 (canstatin) and α3 (tumstatin) chains of type IV of collagen were previously shown to inhibit proliferation of endothelial cells in vitro and suppress tumor growth in vivo (Figure 19). Previous studies showed that the C-terminal 185-203 amino acid fragment of tumstatin, the NC1 domain of α3 (IV) collagen, specifically inhibited the proliferation of melanoma cells. (Maeshima et al., 2001; Han et al., 1997), and its antiangiogenic activity was localized at the N-terminal 54-132 amino acids, whereas the full-length tumstatin specifically inhibited endothelial cells proliferation with no effect on other tumor cells, and also supressed tumor growth of human renal and prostate carcinoma (Maeshima et al., 2000, 2001).Similarly, canstatin, the NC1 domain of a2 (IV) collagen, can also specifically inhibit proliferation of endothelial cells, and

suppress in vivo growth of large and small sizes tumors (Kamphaus et al., 2000).

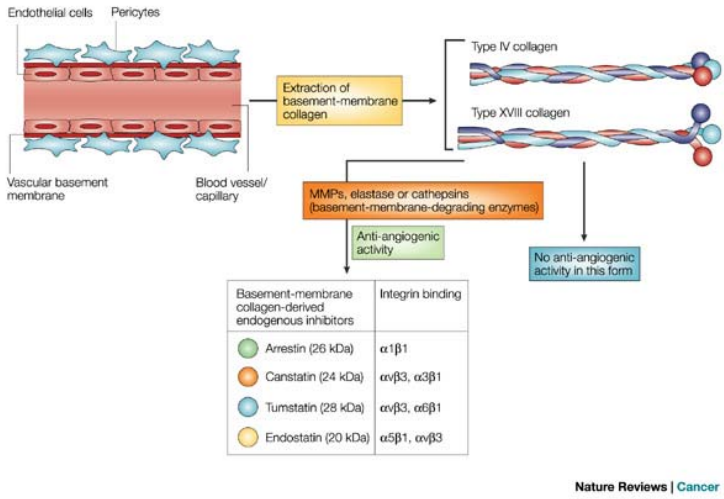


Figure 19: arretin, constatin, tumstatin and endostatin formation.Arrestin, constatin and endostatin derive from the NC1 domain of different a(IV) chains, while endostatin is a fragment of type XVIII of collagen.

As canstatin and tumstatin belong to the NC1 domains of type IV of collagen and have similar biological activities, the amino acid sequences of canstatin and tumstatin were compared and found that they share three homologous regions: the N-termianl 1-89 amino acids (N region), the middle 101-135 amino acids (M region), and the C-terminal 157-197 amino acids , and the final 10 amino acids (C region). Angiogenesis is dependent on specific endothelial cell adhesive events mediated by $\alpha_v\beta_3$ integrin, suggesting that the anti angiogenic activity of endogenous NC1 domain of type IV of collagen (i.e. tumstatin , arrestin and canstatin) takes place through the distruption of the interaction of proliferating endothelial cells with the matrix component such as vitronectin and fibronectin, eventually leading to an important anti-apoptotic signal (Isik et al., 1998, He et al., 2003).

Although, it is well known that gelatinases are involved on VBM degradation during tumor spreading, it's not clear the function and the mechanism by which these two matrix metalloproteinases are involved.

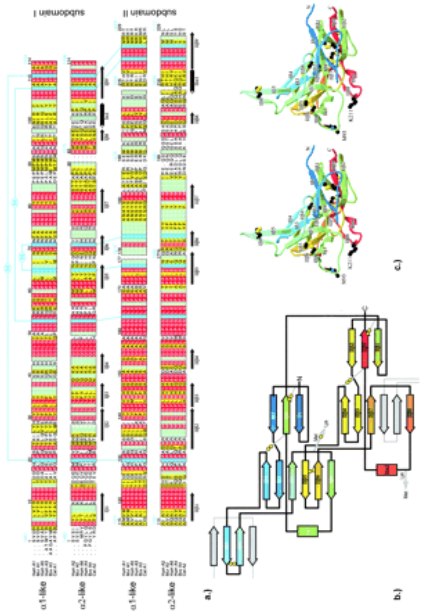


Figure 20: Primary, secondary and tertiary structure of the chains monomers. (*a*) Structure-based sequence alignment of the human NC1(IV) α1 and α2 chains with homologous sequences, grouped into α1-like (human α1, α3, α5, murine α1, bovine α3, and *Caenorhabditis elegans* α1) and α2-like sequences (human α2, α4, α6, murine α2, bovine α4, and *C. elegans* α2). Similar or identical amino acids within each group are colored in yellow and green, whereas residues completely conserved between both groups are shown in red. The sequence numbering (black) following the conventional NC1 numbering (15) is correlated with the total sequence numbering for human α1(IV) and α2(IV) chains (GenBank accession nos. P02462 and P08572; blue). The figure was made with ALSCRIPT (33). (*b*) Topological diagram of the α1-1 subunit of collagen type IV, orientated similar to Fig. 1. The spectral color coding is according to sequential order, from violet (N terminus) to red (C terminus), with disulfides (double circles) being explicitly shown. The β-strands provided from adjacent chain monomers to complete the six-stranded β-sheets are shown in gray. (*c*) Stereo ribbon plot of chain A α1-1. Color coding is as in *b*.

The aim of the study

Understanding the structure and function of the Gelatinases (MMP-2 , MMP-9) active site and exosite domains during type IV of collagen degradation will provide fundamental information on the mechanistic action of gelatinases in the extracellular compartment and on the cell surface. Basic research these themes is applied to the understanding of the pathogenesis of cancer and of chronic inflammatory diseases such as arthritis, with important consequences on the molecular diagnosis.

Type IV of Collagen is a fibrous protein, which is the major component of basement membrane. Under physiological condition, MMP2 and MMP-9 , play a central role in type IV of collagen turnover; infact, the loss of their regulation it is also associated to pathological processes, such as tumours and inflammations.

Although the degradation of type IV of collagen by gelatinases has been extensively studied, it is still far from being understood the mechanism by which this degrading enzymes cleave it.

MMPs have a characteristic multi-domain structure: the gelatinases consist of a catalytic domain linked via a short hinge peptide to the hemopexin-like domain, and of an additional fibronectin-like domain inserted in a loop of the catalytic domain.

The hemopexin-like domain was believed to work as a structural domain bounding substrates, however in recent years it has been shown to play a modulation in positioning the macromolecular substrates during the proteolytic degradation (knauper et al., 1992; Gioia et al., 2002).

In our study, we have investigated the functional properties of human gelatinase A MMP-2 and gelatinase B MMP-9, to process type IV of collagen. In particular, we have characterized catalytic parameters (i.e.: k_{cat}/K_m, k_{cat} and K_m,) for the MMP-2 and MMP-9 proteolytic processing of collagen IV, with the aim to better understand the role of these different gelatinases during tumour progression.

Both enzymes possess the capability to process type IV of collagen, however, the mechanism to recognize and to cleave it, it is not known.

Once the molecular determinants, which are required for the proteolytic process to happen, are identified, they could be targeted by next generation of specific inhibitors.

In order to have a better insight of the molecular determinant underlying on the specificity of the cleavage of this natural substrate by MMP-2 and MMP-9, we have carried a quantitative comparison of the degradation of different chains of type IV of collagen:

i) by whole MMP-2, ii) by whole MMP-2 in the presence of the fibronectin like domain of the enzyme or CBD iii) by MMP-9, iv) by MMP-9 in the presence of the fibronectin like domain of MMP-2 (CBD).
Moreover we have tested the capability of interaction of gelatinases during type IV of collagen degradation. So we have carried out an ex vivo experiment based on a cells migration assay through a type IV of collagen membrane. We used neutrophil cells for their capability to express high quantity of MMP-9 during infiammatory processes. The analysis of our results allows us to state that gelatinases are involved on cell migration during tumour progression and inflammatory diseases on type IV of collagen cleavage. MMP-2 and MMP-9 are able to cooperate each other during collagen degradation, in order to allow endothelial cells to cross the vascular basal membrane and to migrate into a perivascular space where they generate new blood vessels. Infact we have seen that, the inactive MMP-2 bound to type IV of collagen, increased MMP-9 catalytic efficiency against this type of collagen. On the other hand, MMP-2 alone is able to create type IV of collagen fragment with an anti- angiogenic activity. Therefore, we can conclude that, blocking the activity of MMP-2 or MMP-9 with a selective inhibitor may be not enough to reduce the gelatinase induced tumour progression.

Results

We have carried out a kinetic and thermodynamic investigation on the mechanism by which gelatinases process type IV of collagen . In particular, we have derived catalytic parameters k_{cat}, K_m and k_{cat}/K_m, for two different species of gelatinases (namely MMP-2 and MMP-9).
In order to have a better insight into the role of gelatinases domains in modulating the enzymatic activity we have compared type IV of collagen proteolysis by human gelatinase A (MMP-2) and human gelatinases B (MMP-9); aiming to clarify the role of each gelatinase during basement membrane processing which may be important for cellular migration.

I) Degradation of type IV of collagen from human placenta by MMP-2

Figure 1A shows the electrophoretic pattern, under reducing condition, of the enzymatic processing of collagen type IV at 37°C and pH 7.2 as a function of the incubation time with MMP-2. It is possible to observe that only three species are progressively cleaved by MMP-2, namely those characterized by molecular mass of 207, 169 and 92 kDa, respectively, whereas the other species do not seem to be affected by the cleavage event.

Considering that the structure of type IV of collagen is very complex, it is not so easy to identify the different species involved in the final structure.
However, the three species enzymatically processed by MMP-2 have been identified with a mass spectrometry analysis in collaboration with Chris Overall Lab, University of Vancouver, Canada. On the basis of sequence-based mass spectrometry determination we have obtained that the 92 kDa species is a fragment of the α2 chain, due to pepsin digestion, which lacks of the 7S domain at the amino-terminal of the polypeptide. The 207 and the 169 kDa species are likely referrable instead to the total α1-chain and the α1-chain without the NC1 domain, respectively (Soininen, R. et al., 1987; Hostikka, S.L.A. and Tryggvason, K. 1987 .)

It is important to underline that the enzymatic processing of these three species appears to follow the Michaelis-Menten mechanism, as from the linearity of the reciprocal plot for the velocity vs substrate concentration (see Fig. 2). From catalytic parameters reported in Table 1, MMP-2, also called 72 kDa type IV collagenase, cleaves the α-1 (207 kDa) and α-1 chain fragment (169 kDa) with a similar catalytic efficiency (as represented by k_{cat}/K_m), while the processing of the triple helical part of α-2 chain (92 kDa) takes place with an efficiency lower by one order of magnitude. However, if we dissect the overall enzymatic activity, splitting the contribution arising from the substrate recognition (grossly referrable to K_m) from the speed of

the rate-limiting step (i.e., k_{cat}), the difference between the mechanism by which whole MMP-2 processes the three species becomes even more drastic (see Table 1). Thus, MMP-2 shows a very high affinity for the recognition sites on the whole α-1 chain and the α-1 chain fragment (as from the very low values of K_m, see Table 1), but an about 100-fold lower affinity for the triple helical part of α-2 chain. On the other hand, the rate-limiting step for the proteolytic cleavage turns out to be much faster for the triple helical part of α-2 chain (92 kDa) than for the other two species (see Table 1). It must be also stressed that our kinetic analysis has been limited to the first cleavage step of the three components of collagen IV, since this is the only process which follows the Michaelis-Menten approximation and which can be analysed quantitatively. Fragments of lower molecular weight indeed are formed (as also from data reported in Fig. 1), but their kinetic building up cannot be followed appropriately with our detection methods.

II) Degradation of collagen type IV from human placenta by cdMMP-2

Figure 1B shows the electrophoretic pattern, under reducing condition, of the enzymatic processing of type IV collagen at 37°C and pH 7.2 as a function of the incubation time with cdMMP-2.

The catalytic domain of MMP-2 cleaves the same three species of the whole enzyme, following as well the Michaelis-Menten mechanism (see Fig. 2), but, as we can see in Table 1, catalytic parameters are clearly different. In fact, the lack of the hemopexin-like domain leads to a much lower value for k_{cat}/K_m, with respect to the whole enzyme for the α-1 chain and the α-1 chain fragment (at least by one order of magnitude), whereas no significant effect is observed for the catalytic efficiency toward the triple helical part of α-2 chain (see Table 1). However, when we focus on values of k_{cat} and K_m some peculiar differences are observed for the various species with respect to the whole enzyme. Thus, in the case of the total α-1 chain the lower catalytic efficiency appears to be mostly due to a decreased k_{cat}, whereas K_m is only barely increased; on the other hand, for the α-1 chain fragment missing the NC1 domain the ten fold decrease of k_{cat}/K_m is evenly distributed between a three-fold decrease of k_{cat} associated to a three-fold increase of K_m (see Table 1). In the case of the triple helucal part of α-2 chain, the closely similar catalytic efficiency by cdMMP-2 with respect to the whole enzyme corresponds to a very modest two-fold increase of k_{cat} compensated by a similar increase of K_m. As a whole, these results indicate that the hemopexin-like domain of MMP-2 is not playing a crucial role in modulating the enzymatic action of gelatinase A on collagen type IV from human placenta, even though some variation in the catalytic parameters (see Table 1) indeed suggests that the hemopexin-like domain is participating into the determination of some auxiliary processes during the proteolytic processing.

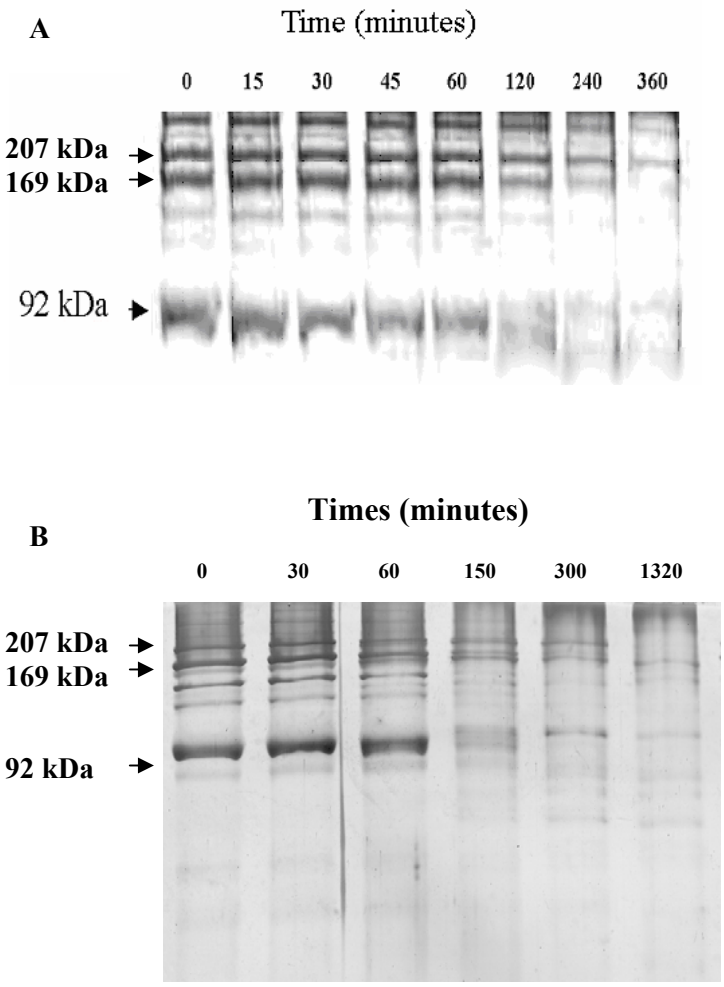


Figure 1: **(panel A)** SDS-PAGE electrophoresis of the processing of collagen type IV from human placenta by whole MMP-2 as a function of time (as indicated) at 37°C and pH 7.1. Fragments under investigation are indicated by their molecular mass: α1 chain of 207 kDa, α1 chain fragment of 169 kDa and triple helical part of α2 chain of 92 kDa. **(panel B)** SDS-PAGE electrophoresis of the processing of collagen type IV from human placenta by the catalytic domain of MMP-2 as a function of time (as indicated) at 37°C and pH 7.1. Fragments under investigation are indicated by their molecular mass: α1 chain of 207 kDa, α1 chain fragment of 169 kDa and triple helical part of α2 chain of 92 kDa . For further details, see text.

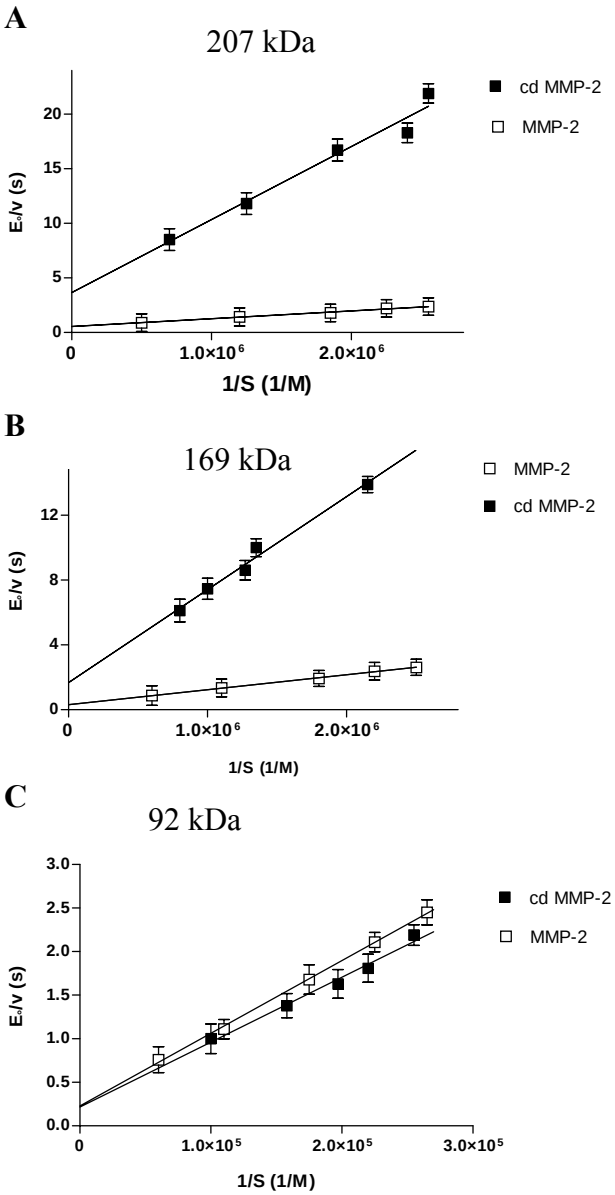


Figure 2: Lineweaver-Burk plots of the processing by whole MMP-2 (□) and by the catalytic domain of MMP-2 (■) of different fragments of collagen type IV from human placenta, namely those characterized by 207 kDa **(panel A),** 169 kDa **(panel B)** and 92 kDa **(panel C)** molecular mass Catalytic parameters are reported in Table 1.

TABLE 1

Catalytic parameters for the enzymatic processing at 37°C and pH 7.1 of collagen type IV from human placenta by the whole MMP-2 and by the catalytic domain of MMP-2 .

	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (M)
Collagen type IV from human placenta			
207 kDa (α1 chain)			
Whole MMP-2	$1.4(\pm0.10)\times10^{6}$	1.76(±0.18)	$1.25(\pm0.13)\times10^{-6}$
Catalytic Domain MMP-2	$1.5(\pm0.10)\times10^{5}$	0.3(±0.04)	$2.0(\pm0.2)\times10^{-6}$
169 kDa (α1 chain fragment)			
Whole MMP-2	$1.1(\pm0.1)\times10^{6}$	3.0(±0.3)	$2.7(\pm0.30)\times10^{-6}$
Catalytic Domain MMP-2	$1.5(\pm0.1)\times10^{5}$	1.2(±0.1)	$7.5(\pm0.6)\times10^{-6}$
92 kDa (triple helical part of α2 chain)			
Whole MMP-2	$1.1(\pm0.1)\times10^{5}$	17.00(±0.16)	$1.55(\pm0.12)\times10^{-4}$
Catalytic Domain MMP-2	$1.2(\pm0.1)\times10^{5}$	31.00(±3.2)	$2.56(\pm0.20)\times10^{-4}$

III) Degradation of native collagen type IV from murine EHS sarcoma by whole MMP-2

The enzymatic processing of the native collagen type IV by whole MMP-2 is shown in Figure 3A at physiological temperature and pH as a function of the incubation time with the enzyme. The electrophoretic pattern shows a much larger number of species than in collagen type IV from human placenta (see Fig. 1), probably due to the higher complexity of the native collagen type IV from the murine Engelbreth-Holm-Swarm (EHS) sarcoma, as also reported by others (Mackay, A.R., et al., 1990).

However, also in this case, only three species (namely those corresponding to a 225, 195 and 169 kDa molecular mass, respectively) appear to be cleaved to a significant amount by whole MMP-2, while the other species with a lower molecular weight appear intact over the time range of the kinetic observation.

Also in the case of native collagen type IV we observe a behaviour compatible with the Michaelis-Menten mechanism (see Fig. 3B) and the catalytic parameters of the enzymatic processing are reported in Table 2. As immediately obvious from data in Table 2, for all three species the catalytic cleavage efficiency by whole MMP-2 is drastically lower than for species from collagen type IV from human placenta (see Figs. 1A and Table 1). In particular, for all three species the substantially reduced enzymatic activity seems mostly attributable to a dramatic decrease of k_{cat} values and only to a lesser extent to an increase for K_m, suggesting that the likely higher structural complexity of this type of collagen IV renders the all network much less susceptible to be cleaved, even though the interaction of whole MMP-2 with native collagen type IV remains characterized by a relatively high affinity (see Table 2).

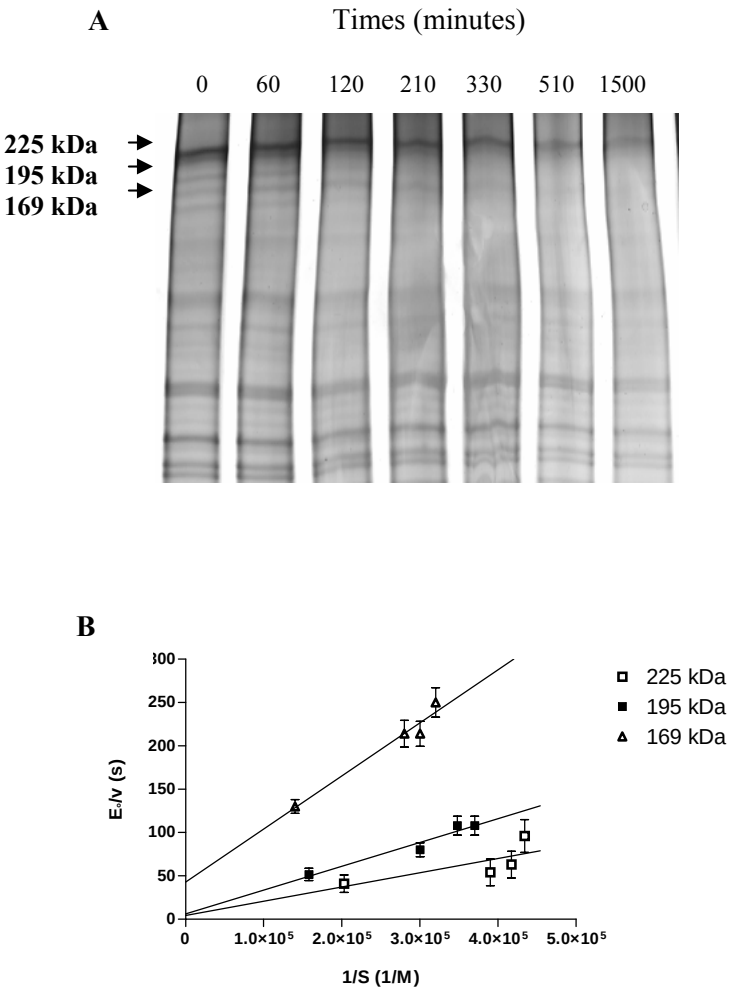


Figure 3: **panel A** SDS-PAGE electrophoresis of the processing of native collagen type IV from murine Engelbreth-Holm-Swarm (EHS) sarcoma by whole MMP-2 as a function of time (as indicated) at 37°C and pH 7.1. Fragments under investigation are indicated by their molecular mass.. **panel B** Lineweaver-Burk plots of the processing by whole MMP-2 of different fragments of native collagen type IV from murine Engelbreth-Holm-Swarm (EHS) sarcoma, namely those characterized by 225 kDa (□), 195 kD (■) and 169 kDa (Δ) molecular mass. Catalytic parameters are reported in Table 2.

TABLE 2

Catalytic parameters for the enzymatic processing at 37°C and pH 7.1 of native collagen type IV (EHS) by whole MMP-2.

	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (M)
Native type IV of collagen (EHS)			
225 kDa MMP-2	$5.0(\pm0.4)\times10^{3}$	0.1(±0.07)	$1.9(\pm0.20)\times10^{-4}$
195 kDa MMP-2	$3.3(\pm0.2)\times10^{3}$	0.2(±0.01)	$5.0(\pm0.4)\times10^{-5}$
169 kDa MMP-2	$1.7(\pm0.1)\times10^{3}$	0.02(±0.001)	$1.0(\pm0.1)\times10^{-5}$

IV) Role of MMP-2 on the migration of neutrophils across a type IV of collagen coating membrane

Figure 4 shows the extent of migration of neutrophils across a type IV collagen coating in the absence of any agent (*column 1*), in the presence of only LPS (as a chemoattractant factor) at a final concentration of 0.5 μg/mL (*column 2*), in the presence of Ilomastat (a wide range inhibitor of MMPs) at a final concentration of 100 μM (*column 3*), in the presence of both 0.5 μg/mL LPS and of active MMP-2 at different concentrations (*columns 4-8*) and in the presence of both 0.5 μg/mL LPS and the product of collagen IV digestion by 40 nM MMP-2 (*column 9*). It is important to underline that neutrophils extracts do not contain appreciable amounts of MMP-2 activity, as from the gelatin zymographic analysis (see inset in Fig. 4), but only MMP-8 collagenase activity and MMP-9 gelatinase activity. As a matter of fact, data reported in columns 2 and 3 of Fig. 4 indicate that i) gelatinase activity is absolutely required for the neutrophil migration, as from the marked inhibitory effect exerted by Ilomastat (which is an inhibitor of MMPs, see ref. [34]), ii) Ilomastat, at concentrations used, does not inhibit MMPs production and it does not exert any toxic activity on neutrophils. The complete inhibition of MMPs produced during experiments in the presence of Ilomastat was confirmed by fluorimetric experiments performed using cell culture supernatant (data not shown). It should be underlined that Ilomastat inhibition is not irreversible since during sample incubation with SDS the inhibitor is removed from MMPs that in turn become able to degrade gelatin during zymography. However, even more interesting is the effect observed if we add active MMP-2, since these data seem to support the idea that the presence of increasing amounts of active MMP-2 is depressing the migration of neutrophils across the type IV collagen coated membrane (which is an oversimplified model for a basement membrane)**.** The mechanism underlying such a behaviour can be explained by the production of lower molecular weight fragments of collagen IV (consequent to the cleavage by MMP-2, see Fig. 1), which impairs the migration of neutrophils. This hypothesis finds support in data reported in *column 9* of Fig. 4, where the LPS-enhanced neutrophil migration (see *column 2* of Fig. 4) is dramatically inhibited by the addition of digestion products of collagen IV by MMP-2.

Similar results have been obtained in the case of membrane coated with native type IV collagen from EHS murine sarcoma, even though in this case

all processes are much less evident because of a greater difficulty of neutrophils to migrate across this membrane coating (data not shown).

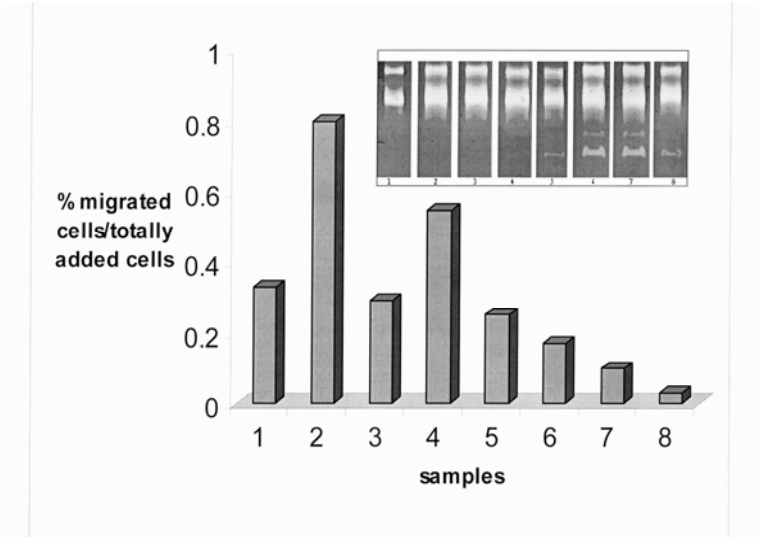


Figure 4 : Neutrophil chemotaxis through empty transwell clots prepared with the indicated type IV collagen concentration was measured as described in Materials and Methods. The number of cells migrated into the lower chamber containing control buffer **(*column 1*)**, in the presence of 0.5 µg/mL LPS **(*column 2*)**, in the presence of 0.5 µg/mL LPS, 1 nM MMP-2 and 100 µM Ilomastat (*column 3*), in the presence of 0.5 µg/mL LPS and of active MMP-2 at different concentration (namely 1 nM in ***column 4*****, 3** nM in ***column 5*****,** 10 nM in ***column 6*** and 40 nM in ***column 7***) and in the presence of 0.5 µg/mL LPS and the product of collagen IV digestion by 5 nM MMP-2 (***column 8***) was determined using an LDH assay as described. The results are presented as a percentage of migrated cells vs. total cells.

(inset) Zymography of the neutrophils supernatant. Different lanes refer to the analysis of the supernatant for the conditions reported in the corresponding columns of the main figure. Pro-MMP-9 (pMMP9), MMP-9 (MMP9) and MMP-2 (MMP2) are indicated on the right side of the inset.

V) Role of the fibronectin like domain on the processing of type IV of collagen from human placenta by whole MMP-2

The SDS-PAGE electrophoretic pattern in Figure 5 shows the role of rCBD, a recombinant form of the fibronectin like domain, during the enzymatic processing of collagen type IV from human placenta by whole MMP-2. It appears evident as the presence of rCBD (to a final concentration of 50 μM) mostly inhibits the proteolytic activity of MMP-2 on all three species of collagen type IV susceptible to cleavage. It clearly demonstrates that the affinity of rCBD is comparable to that of whole MMP-2, suggesting that the actual binding site for MMP-2 on all three species of collagen type IV from human placenta takes place through the fibronectin-like domain of MMP-2.

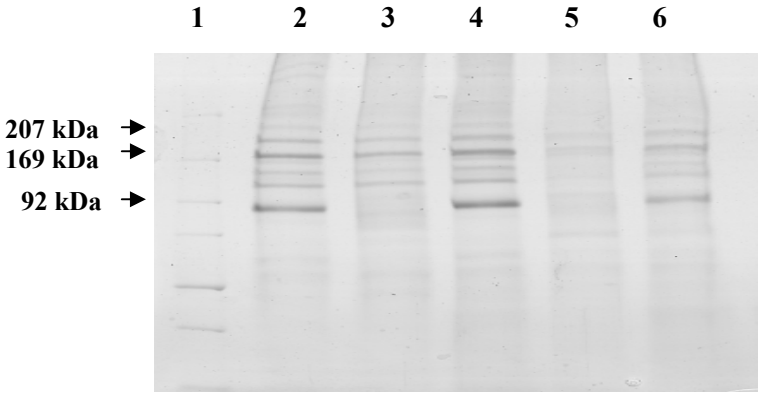


Figure 5: SDS-PAGE electrophoresis of the processing of type IV collagen from human placenta by whole MMP-2 in the absence (lanes 3 and 5) and in the presence of 50 μM rCBD (lanes 4 and 6) at two different intervals after exposure to whole MMP-2, namely 30 min. (lanes 3 and 4) and 45 min. (lanes 5 and 6). Lane 1 corresponds to molecular weight markers and lane 2 is intact collagen type IV from human placenta.

VI) Degradation of type IV of collagen from human placenta by MMP-9

Figure 6A shows the electrophoretic pattern, under reducing condition, of the enzymatic processing of collagen type IV from human placenta at 37°C and pH 7.1 by MMP-9. We can observe that MMP-9 cleaves the same three species of type IV of collagen process by MMP-2; characterized by a molecular mass of 207, 169 and 92 kDa respectively. Also in this case, the 207 and the 169 kDa species are referrable to the α−1 chain and α−1 chain fragment, respectively, on the basis of sequence-based mass determination in collaboration with Prof. Overall Lab, University of Vancouver, Canada (Hostikka and Tryggvason, 1987; Soininen et al., 1987). Further, the 92 kDa fragment corresponds to the triple helical part of the α-2 chain of human collagen IV, with amino-termini at positions 675 and 676 (occuring as a mixture), suggesting that this species is a fragment due to pepsin digestion.

However, it is important to underline that the enzymatic processing of these three species appears to follow the Michaelis-Menten mechanism, as from the linearity of the reciprocal plot for the velocity vs. substrate concentration (see Figure 6B). In Table 3 we show the catalytic parameters of the cleavage event. These data indicate that the catalytic efficiency of MMP-9 is higher on the 92 kDa species (as from k_{cat}/K_m values, see Table 3) than for the others. On the other hand, the rate-limiting step for the proteolytic cleavage (indicated by k_{cat} values) turns out to be higher for the α-1 chain than for the others. Further, the substrate affinity (referable to the K_m value, see Table 3) is much higher for the triple helical part of α-2 chain and α-1 chain fragment than for the α-1 chain.

As a whole, these data indicate that MMP-9 interacts more tightly with α-1 chain fragment than with the α-1 chain, which is instead cleaved more easily, displaying a 10-fold faster rate-limiting step (see Table 3). Interestingly, the triple helical part of α-2 chain appears to be processed in a fashion closely similar to that displayed by the α-1 chain fragment (see Table 3).

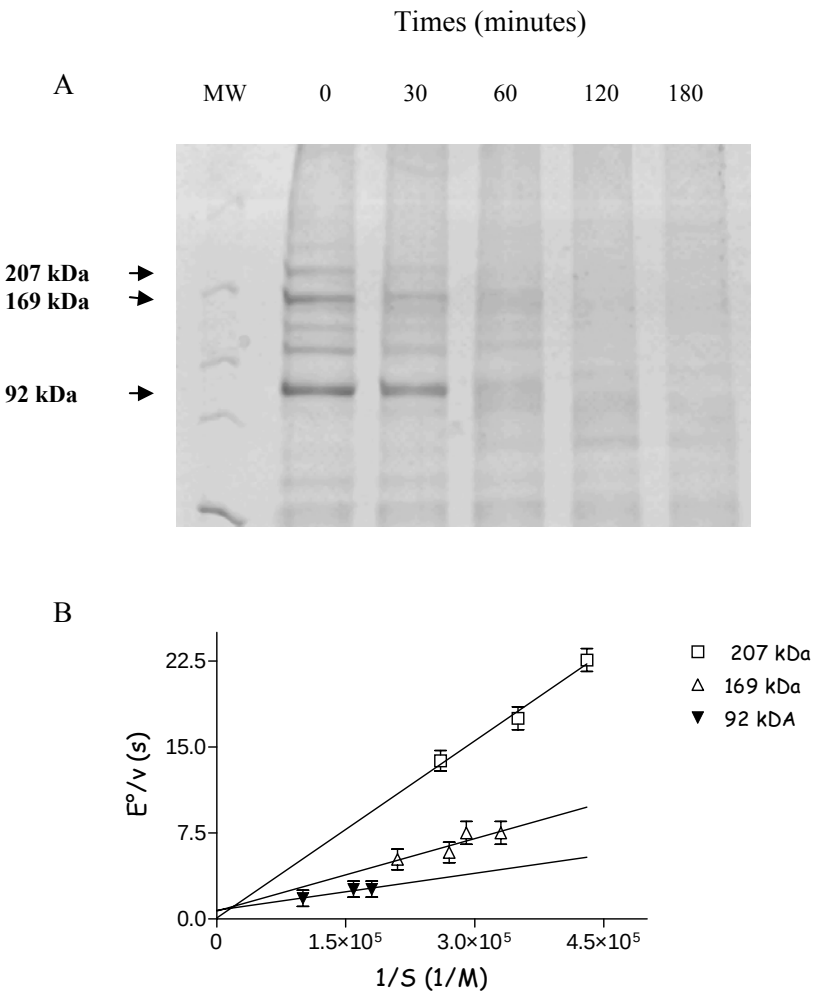


Figure 6: **panel A** ,SDS-PAGE electrophoresis of the processing of collagen type IV from human placenta by MMP-9 as a function of time (as indicated) at 37°C and pH 7.1. Left column reports broad range molecular weight markers. Fragments under investigation are indicated by their molecular mass. **panel B** Lineweaver-Burk plots of the processing by MMP-9 of different fragments of collagen type IV from human placenta, namely those characterized by 207 kDa (□), 169 kDa (Δ) and 92 kDa (▼) molecular mass. Catalytic parameters are reported in Table 3.

VII) Degradation of native collagen type IV from murine EHS sarcoma by whole MMP-9

The SDS-electrophoretic pattern in figure 7A shows the enzymatic processing of the native collagen type IV by MMP-9 at physiological temperature and pH as a function of the incubation time with the enzyme. The electrophoretic pattern shows a much larger number of species than in collagen type IV from human placenta (see Figs. 6A and 7A), probably due to the higher complexity of the native collagen type IV from Engelbreth-Holm-Swarm (EHS).

Three species (characterized by 225, 195 and 169 kDa MW) appear to be significantly cleaved by MMP-9, while the other species with a lower molecular weight seem to be resistant to the cleavage event. Also in the case of native collagen type IV we observe a behaviour compatible with the Michaelis-Menten mechanism (see Fig. 7B) and the catalytic parameters of the enzymatic processing are reported in Table 3. The overall catalytic efficiency of MMP-9 is not drastically different for these three species (as from k_{cat}/K_m, see Table 3), even though it seems somewhat less active toward the 195 kDa form and it is significantly lower (by about one order of magnitude) than that for the $\alpha-1$ chain and $\alpha-1$ chain fragment of collagen type IV from human placenta (see Table 3). This behaviour is substantially due to a decrease of k_{cat} values, suggesting that the likely higher structural complexity of the native type of collagen renders the network much less susceptible to be cleaved. However, an exception is represented by the species characterized by 169 kDa MW (present in both substrates and likely referable to the α-1 chain fragment), which shows a much higher affinity for MMP-9 in the native collagen IV from EHS than in that from human placenta (see Table 3); it clearly indicates that in the more complex network the binding site is probably much more structured and suitable for a preferential interaction with MMP-9, likely representing the primary recognition site for MMP-9 on collagen IV.

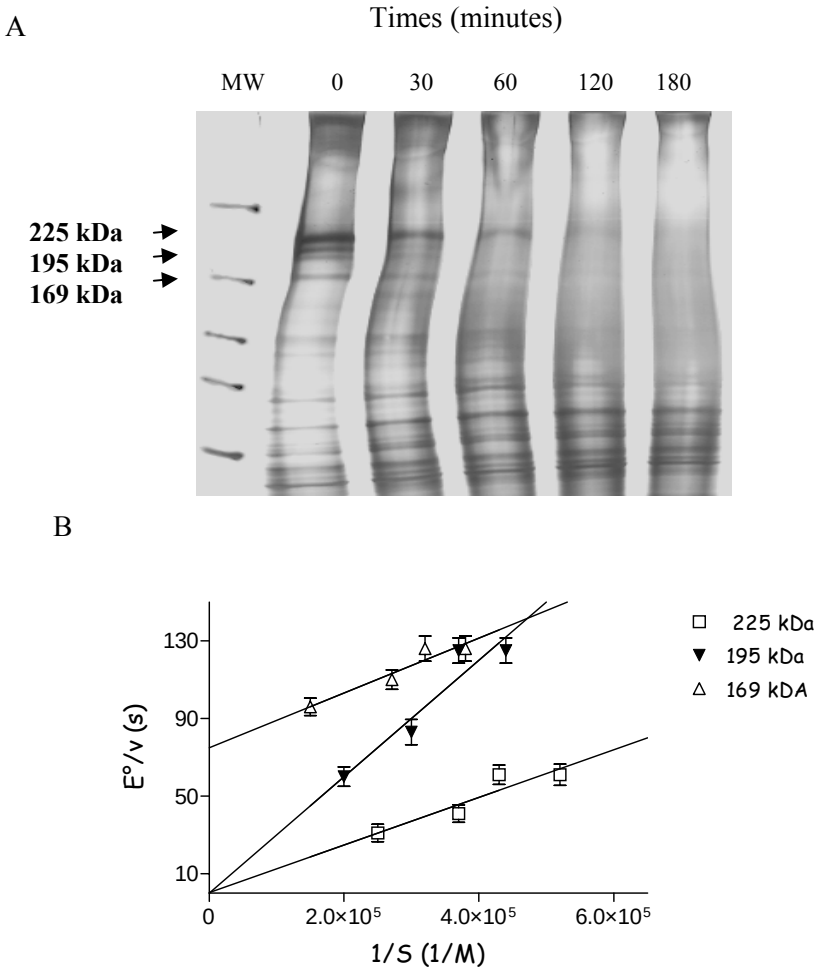


Figure 7: **panel A** SDS-PAGE electrophoresis of the processing of native collagen type IV from murine EHS sarcoma by MMP-9 as a function of time (as indicated) at 37°C and pH 7.1. Left column reports broad range molecular weight markers. Fragments under investigation are indicated by their molecular mass. **panel B** Lineweaver-Burk plots of the processing by MMP-9 of different fragments of native collagen type IV from murine EHS sarcoma, namely those characterized by 225 kDa (□), 195 kDa (▼) and 169 kDa (Δ) molecular mass. Catalytic parameters are reported in Table 2. For further details, see text.

TABLE 3

Catalytic parameters for the enzymatic processing by MMP-9 at 37°C and pH 7.1 of collagen type IV of human placenta and of native collagen IV from murine EHS sarcoma.

	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (M)
Collagen type IV from human palcenta			
207 kDa (α1 chain)	$2.0(\pm0.2)\times10^{4}$	10.2 ± 1.6	$5.2(\pm0.6)\times10^{-4}$
169 kDa (α1 chain fragment)	$4.9(\pm0.5)\times10^{4}$	1.4 ± 0.2	$2.9(\pm0.3)\times10^{-5}$
92 kDa (triple helical part of α2 chain)	$1.3(\pm0.2)\times10^{5}$	1.3 ± 0.2	$1.0(\pm0.1)\times10^{-5}$
Denatured collagen type IV from human placenta			
207 kDa (α1 chain)	$2.0(\pm0.3)\times10^{5}$	4.9 ± 0.6	$2.4(\pm0.4)\times10^{-5}$
169 kDa (α1 chain fragment)		$1.4(\pm0.3)\times10^{5}$	7.8 ± 1.0 $5.6(\pm0.8)\times10^{-5}$
Native type IV of collagen (EHS)			
225 kDa	$8.1(\pm1.0)\times10^{3}$	3.4(±0.3)	$4.2(\pm0.6)\times10^{-4}$
195 kDa	$3.6(\pm0.5)\times10^{3}$	0.7(±0.1)	$1.9(\pm0.3)\times10^{-4}$
169 kDa	$6.1(\pm0.8)\times10^{3}$	$1.4(\pm0.2)\times10^{-2}$	$2.3(\pm0.4)\times10^{-6}$

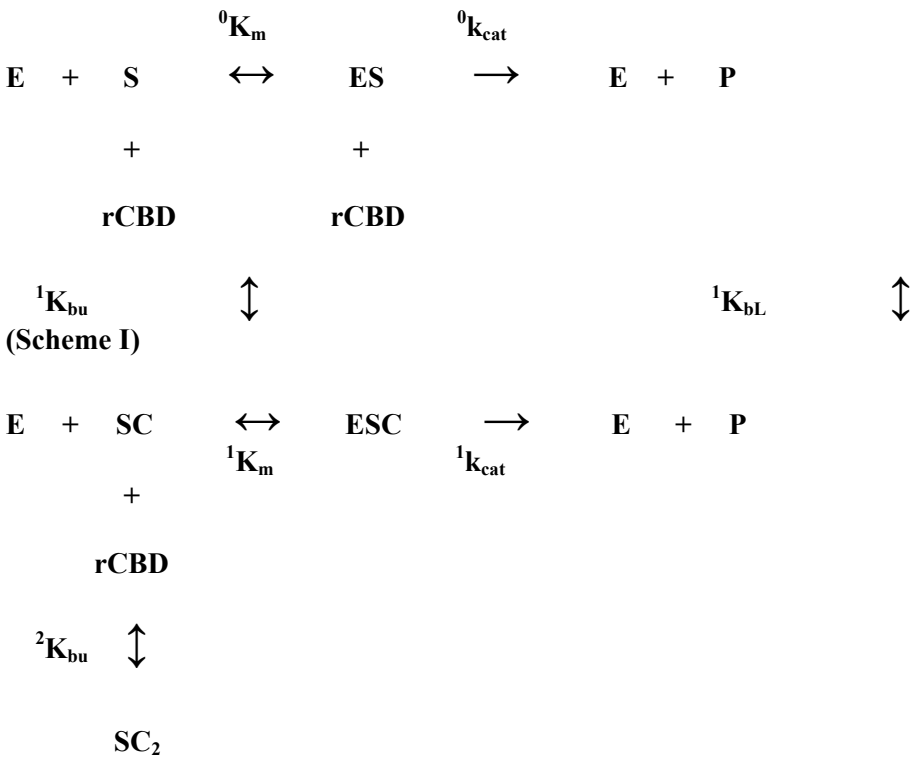
VIII) Contribution of the interaction of fibronectin-like domain of MMP-2 (CBD) to the processing of collagen type IV from human placenta by MMP-9

Figure 8A shows the processing of type IV collagen from human placenta by MMP-9 in the absence and in the presence of 20 μM CBD**,** which is known to bind collagen IV in the micromolar range (Steffensen et al., 1995). In particular, it has been suggested that the CBD domain (also called the fibronectin-like domain) interacts with collagen by means of all three fibronectin type II modules (Briknarova et al., 2001) and that this interaction is likely different from that of other MMPs with collagen I (Tam et al., 2004). It comes out very clearly that at this concentration CBD facilitates the cleavage of the three species by MMP-9. It suggests that (i) the binding site for CBD is different from that for MMP-9 (supporting that also for collagen IV the distinction of binding sites for MMPs is present), and (ii) the binding of CBD brings about a conformational change of type IV collagen chains, which facilitates the enzymatic action of MMP-9 on the same species. In this respect, it is important to outline that a parallel investigation on the catalytic parameters for the α-1 chain and α-1 chain fragment of native and denatured collagen type IV indicates that denaturation brings about a marked increase of the catalytic efficiency by MMP-9 (see Table 4), suggesting that the CBD-induced conformational change might be referable to a gross unwinding of the collagen IV chains.

In order to understand how this phenomenon is exerted we have carried out an extensive quantitative analysis of the enzymatic mechanism of MMP-9 on collagen type IV as a function of the concentration of CBD. Figure 8A shows the dependence of catalytic parameters on the concentration of CBD for the three species investigated (i.e., the 207, 169 and 92 kDa). In general terms, it is evident as at low concentrations (i.e., < 10 μM) CBD induces an increase of the enzymatic efficiency (as from k_{cat}/K_m, see Fig. 8B), which is mostly related to an increase for substrate affinity (that is a decrease of K_m, see Fig. 8C) and a modest increase for the speed of the rate-limiting step (Fig. 8D). At higher concentrations of CBD, we observe a relevant decrease of the enzymatic efficiency (see Fig. 8B), completely referable to a decrease of substrate affinity (see Fig. 8C), whereas the value of the rate-limiting step k_{cat} tends to level off (see Fig. 8D). Such a behaviour indicates the existence of two binding sites for CBD on collagen type IV chains; one is characterized by a higher affinity and it displays positive allosteric features on the binding site of MMP-9, inducing an increase of its enzymatic action.

The second binding site for CBD displays a lower affinity and it exerts a competitive inhibitory effect on the enzymatic action of MMP-9, likely because it closely overlaps with the binding site for MMP-9.

On the basis of these considerations, we have analyzed the dependence of the enzymatic action of MMP-9 on the CBD concentration according to the following scheme



where the first binding step shows the allosteric scheme, implied by the above comments, in which the E (i.e., MMP-9) is able to interact through its binding site with substrate even when it is bound to CBD (i.e., SC), whereas the second binding event, leading to the form SC_2, brings about a competitive inhibition, even though in this case it is the substrate to be inhibited and not the enzyme.

We have adopted this scheme for the analysis of the CBD concentration dependence of the enzymatic action of MMP-9 for all three substrate chains.

The continuous lines in Fig. 8 represent the non linear least-squares fitting of experimental data according to Scheme I, employing the following equations

$$^{obs}k_{cat}/K_m = \frac{(^0k_{cat}/K_m) + (^1k_{cat}/K_m)\cdot {}^1K_{bu}\cdot[rCBD]}{1 + {}^1K_{BU}\cdot[rCBD] + {}^1K_{bu}\cdot {}^2K_{bu}\cdot[rCBD]^2}$$

(Equation 8a)

$$^{obs}K_m = {}^0K_m \cdot \frac{1 + {}^1K_{bu}\cdot[rCBD] + {}^1K_{bu}\cdot {}^2K_{bu}\cdot[rCBD]^2}{1 + {}^1K_{bL}\cdot[rCBD] + {}^1K_{bL}\cdot {}^2K_{bL}\cdot[rCBD]^2}$$

(Equation 8b)

$$^{obs}k_{cat} = \frac{{}^0k_{cat} + {}^1k_{cat}\cdot {}^1K_{bL}\cdot[rCBD]}{1 + {}^1K_{BL}\cdot[rCBD]}$$

(Equation 8c)

where $^0k_{cat}/K_m$ and $^1k_{cat}/K_m$ in Eq. 8a are the values of the catalytic efficiency of MMP-9 toward the substrate in the absence of CBD (i.e., S) and to substrate bound by CBD at the first allosteric binding site (i.e., SC); since Scheme I implies that the second binding site for CBD induces a competitive inhibition $^2k_{cat}/K_m = 0$. $^1K_{bu}$ and $^2K_{bu}$ in Eq. 8a are the association binding constants for CBD to the two sites on the substrate in the absence of the enzyme and they have the same meaning in Eq. 8b, where ^0K_m is the Michaelis-Menten constant in the absence of CBD. $^1K_{bL}$ is the association binding constant for CBD to the first allosteric site on the substrate when the enzyme is bound; Scheme I implies that $^2K_{bL} = 0$. In Eq. 8c, $^0k_{cat}$, and $^1k_{cat}$ are the values of rate for the rate-limiting step of MMP-9 toward the enzyme:substrate complex in the absence of CBD (i.e., ES) and to the enzyme:substrate bound by CBD at the first allosteric binding site (i.e., ESC), respectively; $^1K_{bL}$ has the same meaning as in Eq. 8b and Scheme I implies that $^2K_{bL} = 0$. The outcome of this analysis is reported in Table 4 for the three collagen type IV chains from human placenta investigated and it turns out that upon binding of CBD to its first allosteric site (see Scheme I) the increase of the catalytic efficiency (as from k_{cat}/K_m, see Fig. 8 and Table 4) is only two- or three-fold for the α-1 chain (characterized by 207 kDa MW) and α-1 chain fragment (characterized by 169 kDa MW), whereas it is more marked (i.e., about 100-fold) in the case of the triple helical part of α-2

chain. This effect finds an explanation when we compare $^{1}K_{bu}$ and $^{1}K_{bL}$ (see Table 4), which reflects the long range functional influence between the enzyme binding and CBD binding at the two distinct sites (see Scheme I). Thus, while for α-1 chain and α-1 chain fragment the effect is very modest (being always less than a twofold enhancement, see Table 4), for the triple helical part of α-2 chain we observe a positive cooperative effect with more than a 50-fold increase for CBD affinity to the first site when MMP-9 is bound to its binding pocket. This feature underlies a dramatic conformational change for the triple helical part of α-2 chain (which occurs to a much more limited extent in the other two chains) upon binding of CBD, and this is accompanied by an increase of the speed for the rate-limiting step k_{cat} (see Table 4), which again is more marked for the triple helical part of α-2 chain than for the other two species. However, it worth outlining that the effect exerted by the interaction of CBD with the first binding site (that is the enhancement of the catalytic efficiency of MMP-9, see Table 4) is much smaller than what observed for the denaturation of collagen IV, clearly indicating that the CBD-induced conformational change displays features significantly different from a simple unwinding of the collagenous portion of collagen type IV.

An other peculiar feature of this mechanism is represented by the fact that for both α-1 chain and α-1 chain fragment the binding of CBD to the second site (likely overlapping with that for MMP-9, thus displaying the characteristics of a competitive inhibition) shows a much lower affinity than the first allosteric binding site, while the opposite can be observed for the triple helical part of α-2 chain (see Table 4).

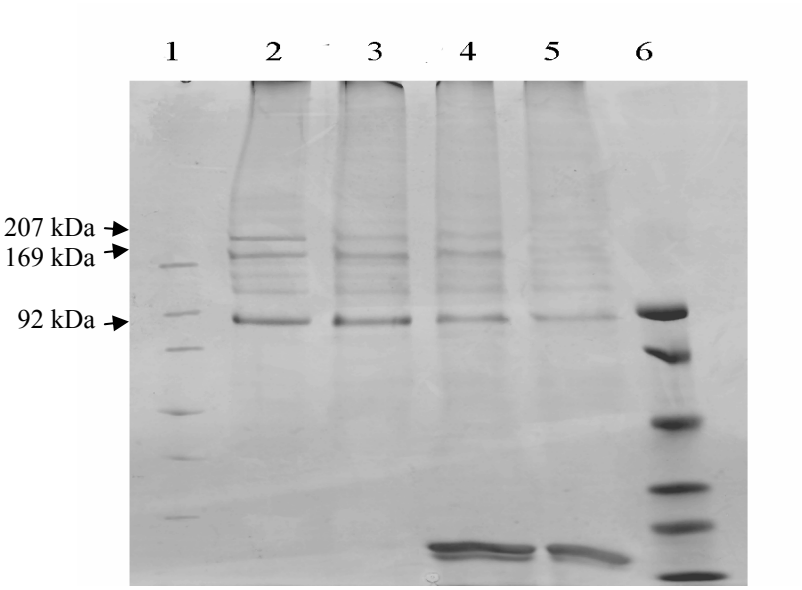


Figure 8A: SDS-PAGE electrophoresis of the processing of collagen type IV from human placenta after 30 min of exposure to MMP-9 at 37°C and pH 7.1 in the absence **(*lane 3*)** and in the presence of 20 μM CBD **(*lane 4*)** and of 5 μM CBD **(*lane 5*)**. *Lane 2* reports collagen type IV from human placenta not exposed to MMP-9. ***Lanes 1*** and ***6*** are broad range and low molecular weight markers, respectively, ***lane 2*** rapresent collagen IV alone.

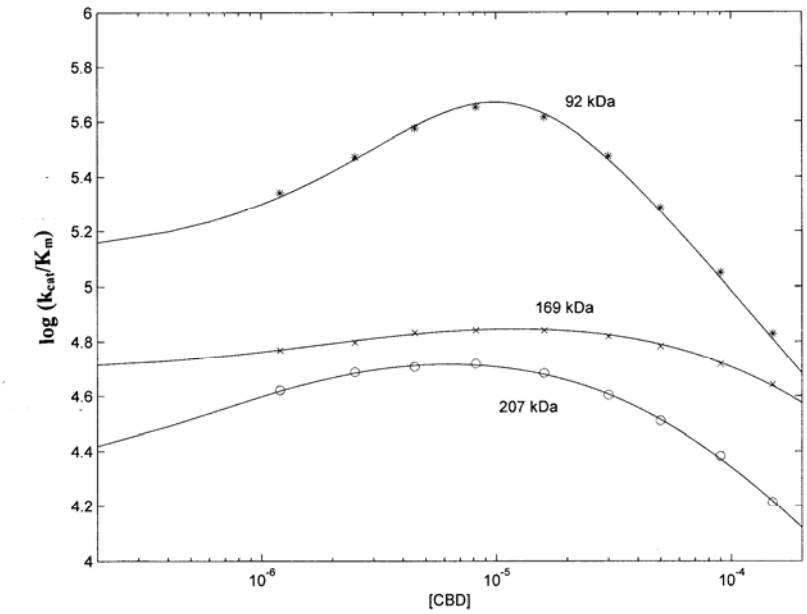


Figure 8B: Dependence on CBD concentration of enzymatic processing of collagen type IV from human placenta by MMP-9 at 37°C and pH 7.1 for k_{cat}/K_m K_m . Different species are indicated in each figure and continuous lines have been obtained by non linear least-squares fitting of data according to Eq. 8a .Parameters employed for each equation are reported in Table 4 or the three species investigated.

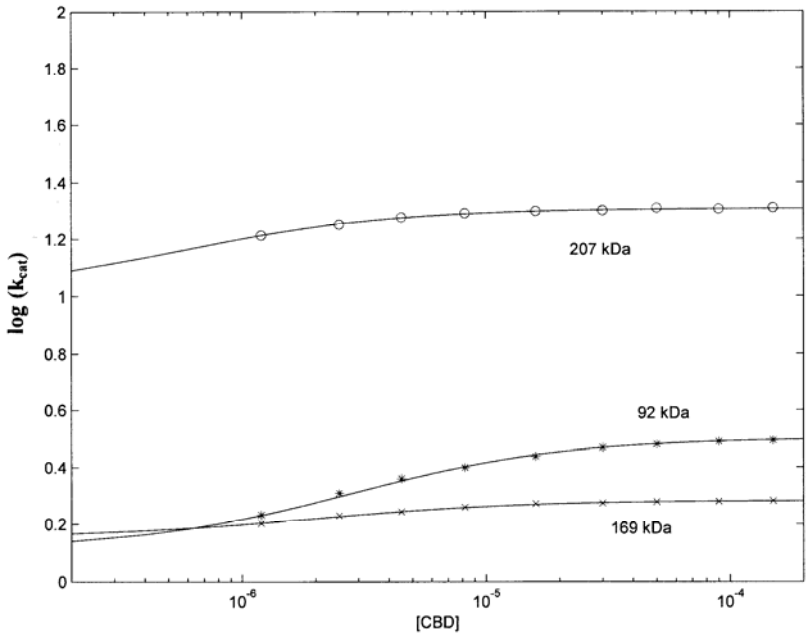


Figure 8C: Dependence on CBD concentration of enzymatic processing of collagen type IV from human placenta by MMP-9 at 37°C and pH 7.1 for K_{cat} . Different species are indicated in each figure and continuous lines have been obtained by non linear least-squares fitting of data according to Eq. 8c.

Parameters employed for each equation are reported in Table 4 or the three species investigated.

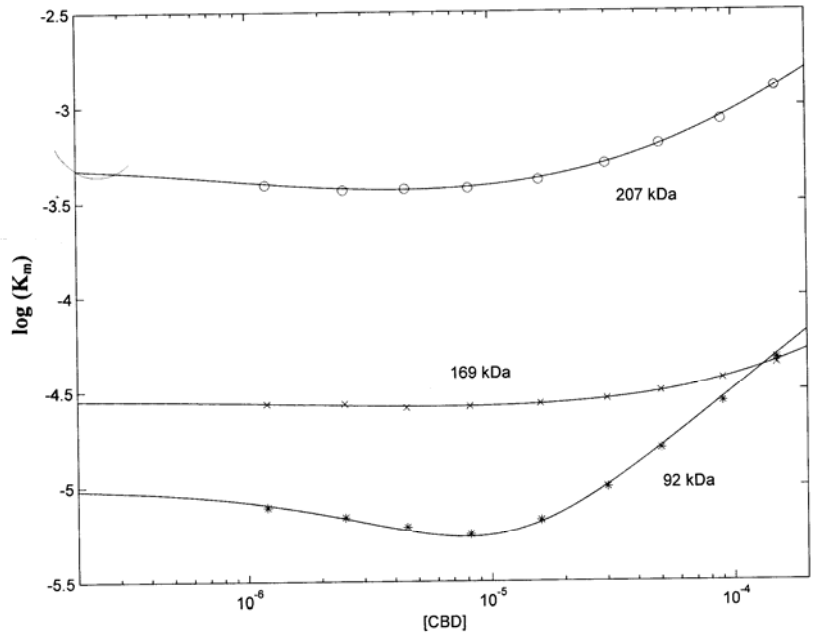


Figure 8D: Dependence on CBD concentration of enzymatic processing of collagen type IV from human placenta by MMP-9 at 37°C and pH 7.1 for k_m. Different species are indicated in each figure and continuous lines have been obtained by non linear least-squares fitting of data according to Eq. 8b . Parameters employed for each equation are reported in Table 4 or the three species investigated.

TABLE 4

Catalytic and binding parameters from the analysis of data according to Scheme I and employing Eqs. 8a, 8b and 8c.

	207 kDa	**169 kDa**	**92kDa**
$^{0}k_{cat}/K_m$ **($M^{-1}s^{-1}$)**	2.0(±0.2) x 10^4	4.9(±0.5) x 10^4	1.3(±0.2) x 10^5
$^{1}k_{cat}/K_m$ **($M^{-1}s^{-1}$)**	6.5(±0.7) x 10^4	7.9(±0.9) x 10^4	1.1(±0.2) x 10^7
$^{0}K_m$ **(M)**	5.1(±0.6) x 10^{-4}	2.9(±0.3) x 10^{-5}	9.9(±1.2) x 10^{-4}
$^{1}K_m$ **(M)**	3.2(±0.4) x 10^{-4}	2.4(±0.3) x 10^{-5}	2.8(±0.4) x 10^{-5}
$^{0}k_{cat}$ **(s^{-1})**	10.2 ± 1.6	1.4±0.2	1.3±0.2
$^{1}k_{cat}$ **(s^{-1})**	20.3 ± 2.4	1.9±0.2	3.2±0.4
$^{1}K_{bu}$ **(M^{-1})**	8.1(±0.9) x 10^5	3.7(±0.4) x 10^5	6.5(±0.7) x 10^3
$^{2}K_{bu}$ **(M^{-1})**	2.0(±0.2) x 10^4	5.6(±0.6) x 10^3	1.1(±0.2) x 10^6
$^{1}K_{bL}$ **(M^{-1})**	1.3(±0.2) x 10^6	4.3(±0.5) x 10^5	2.3(±0.3) x 10^5

IX) Contribution of the interaction of fibronectin-like domain of MMP-2 (CBD) to the processing of native collagen type IV (EHS) by MMP-9

A similar effect of CBD can be observed for the processing of native type collagen from murine EHS sarcoma, as it is shown in Figure 9, where it is evident that the presence of 25 μM CBD brings about a significantly higher catalytic efficiency by MMP-9 on the three species investigated. However, since the affinity for CBD and MMP-9 (as from K_m values, see Table 1) is much lower than for the type IV collagen from human placenta, we have not carried out a similar analysis for the native type collagen IV from murine EHS sarcoma, limiting ourselves to detect a qualitatively similar phenomenon, which allows us to consider this rCBD-linked enhancing effect as a general one.

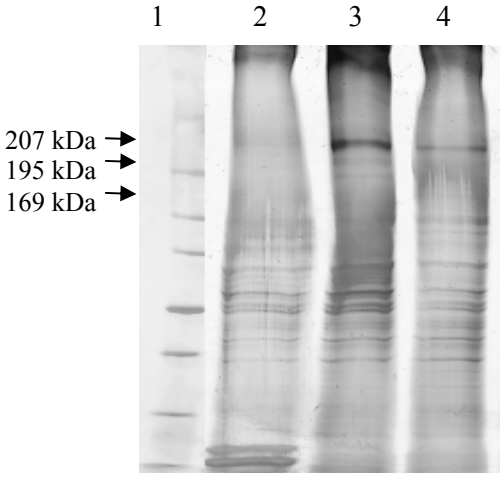


Figure 9: SDS-PAGE electrophoresis of the processing of native collagen type IV from murine EHS sarcoma after 45 min of exposure to MMP-9 at 37°C and pH 7.1 in the absence (*lane 4*) and in the presence of 50 μM CBD (*lane 2*). *Lane 3* reports native collagen type IV from murine EHS sarcoma and the CBD domain not exposed to MMP-9. *Lane 1* reports broad range molecular weight markers.

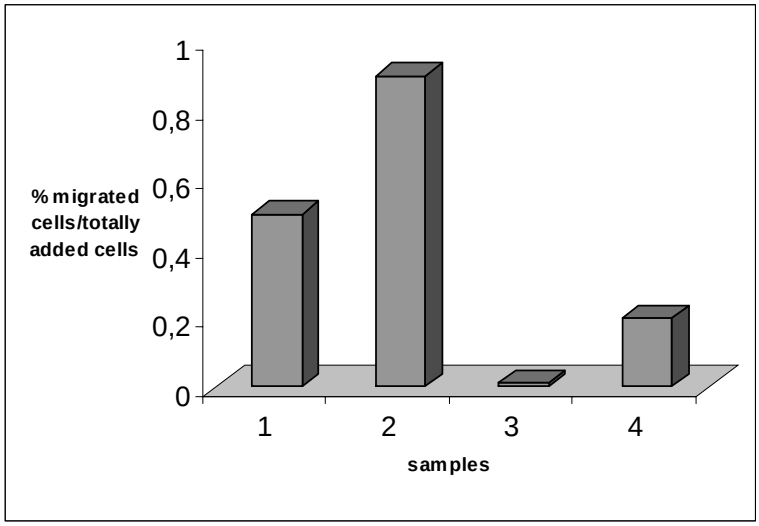


Figure 10: Neutrophil chemotaxis through empty transwell clots prepared with the indicated type IV collagen concentration was measured as described in Materials and Methods. The number of cells migrated into the lower chamber containing positive control *Lane 1*: LPS to a final concentration of 500 ng/ml; *lane 2*: type IV collagen (1 mg/ml); *lane 3*: type IV collagen (1 mg/ml) digested for 6 hours with MMP-2 (60 nM) , after degradation MMP-2 was stochiometrically blocked with Ilomastat, a synthetic MMPs inhibitor; *lane 4*: type IV collagen (1 mg/ml) digested for 6 hours with MMP-9 (60 nM) , after degradation MMP-9 was stechiometrically blocked with Ilomastat, a synthetic MMPs inhibitor

X) Synergistic effect between MMP-2 and MMP-9 during neutrophil cells migration across a coating membrane of collagen type IV

Figure 11 shows the extent of migration of neutrophils across a type IV collagen coating in the absence of any agent (*column 1*), in the presence of only fMLP(as a chemoattractant factor) at a final concentration of 200 nM (*column 2*), in the presence of active MMP-2 at a final concentration of 35 nM (*column 3*), in the presence of 200 nM f-MLP and of a recombinant fibronectin like domain of MMP-2, rCBD-2 at a final concentration of 25 nM (*columns 4*) and in the presence of f-MLP 200 nM and of pro-MMP-2 25 nM (*column 5*), and in the presence of MMP-2 devoid of the catalytic domain, at a final concentration of 50 nM. It is important to underline that neutrophils extracts do not contain appreciable amounts of MMP-2 activity, as from the gelatin zymographic analysis (figure 11), but only MMP-8 collagenase activity and MMP-9 gelatinase activity. As a matter of fact, data reported in columns 2 and 3 indicate that i) MMP-9 activity is absolutely required for the neutrophil migration, while the addition of MMP-2 creates an inhibition of neutrophil migration (column 3). This event is due to the formation of fragments of collagen IV with anti-proliferative activity, formed by the proteolysis of collagen by MMP-2. The behaviour changes when we added to the neutrophil cells solution different biochemical forms of MMP-2.

As we can see on column 4, the presence of f-MLP and rCBD-2, increase the rate of migration of neutrophil cells across the collagen IV. This behaviour is given by the synergic effect between MMP-2 and MMP-9 during the cleavage of collagen IV, as we have already seen on figure 8, 9.The same situation is reported on column 5, where the number of cells migrated is higher than the control on column 1 and 2, in spite of the sobstitution of rCBD with pro-MMP-2 at the same final concentration. The rate of migration, for the most part, has been obtained when we added to the solution f-MLP in presence of MMP-2 without the catalytic domain. In this case, the lack of the catalytic domain on MMP-2 leads to the only bond of the enzyme to collagen IV, while MMP-9 increases its capability to cleave the substrate.

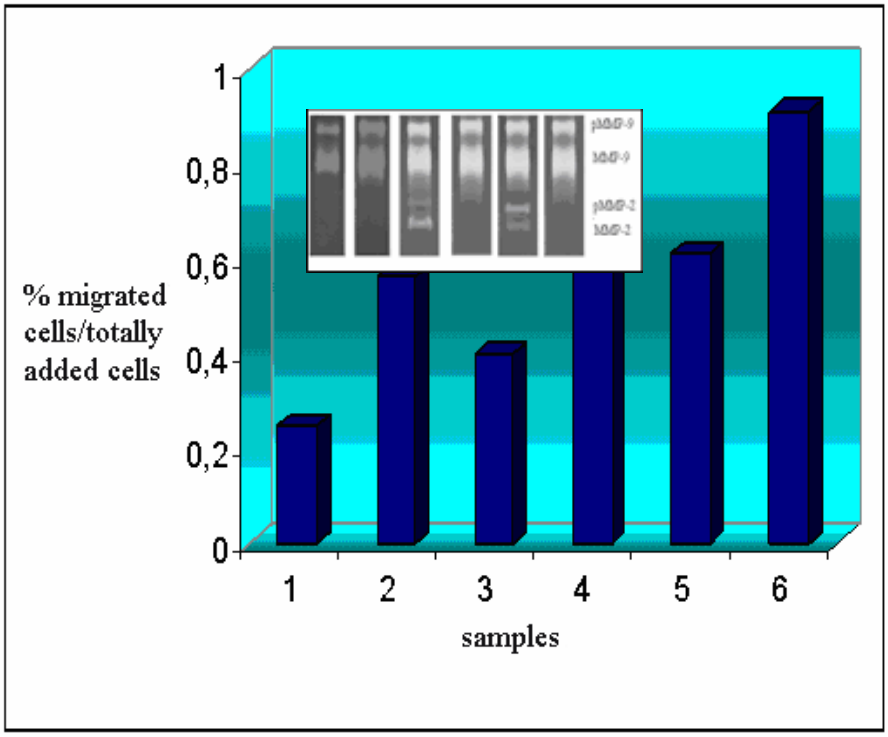


Figura 11: Nrutrophil migration across a coating of collagen type IV in presence of different forms of MMP-2. The columns rapresent the number of cells migrated in presence of a control buffer (column 1), of f-MLP 200 nM (column 2), of MMP-2 35 nM e f-MLP 200 nM (column 3), of rCBD 25 nM e f-MLP 200 nM (column 4), of proMMP-2 25 nM e f-MLP 200 nM (column 5) e of MMP-2 without the catalytic domain 50 nM and of f-MLP 200 nM (column 6). On the top of the figure it is rapresented the zymografy of each samples.

Discussion

It is well known that the macromolecular organization and the biomechanical stability of basement membrane is mainly given by the network of type IV collagen (Kuhnk et al., 1994). Therefore, the mechanism of its proteolytic processing is of the utmost importance for a better comprehension of the tumor cells spreading across the basement membrane as well as of most remodeling and repair processes (Stetler-Stevenson et al., 1993). Previous studies have shown that both collagen type IV from human placenta and native type IV collagen from Engelbreth-Holm-Swarm murine sarcoma (EHS) can be processed by MMP-2 and MMP-9 (Eble et al., 1996). This process is sensibly temperature-dependent, being more efficient at 37°C than at lower temperature (Mackay et al., 1990),probably because of some loosening on the tightness of the network and of the triple-helical assembly upon temperature rising (Tanjore et al.,2001). Moreover, MMP-2 has a possible direct role for the modulation of the endoproteolytic process of the glomerular basement membrane responsible for the pathogenesis of the Alport's syndrome (Xu et al., 2005).

However, up to now no attempt has been carried out to correlate the integrity of collagen IV (and of other components of the basement membrane) with the capability of cells to migrate across the basement membrane for different physiological and pathological processes, such as inflammatory response, angiogenesis, tumor dissemination and others. This correlation can only be made by associating (i) the detailed mechanism by which a MMP is cleaving and fragmenting different chains of collagen IV (and of other components), (ii) its molecular control and modulation by environmental conditions and (iii) the effect of these events on the cellular migration.

We present a clearcut evidence that gelatinase activity by MMP-9 indeed is important for the migration of neutrophils across a barrier represented by type IV collagen and that inhibition of its activity (upon addition of Ilomastat, a MMP inhibitor, see ref. (Steffensen et al., 2002) leads to a great depression of neutrophils migration (see Fig. 4). However, this observation does not represent an absolute novelty, since previous papers suggested the importance of this role (Declaux et al., 1996).On the other hand, a partially unexpected and very novel result is represented by the observation that MMP-2 plays a strong negative action on this process (see Fig. 4). Therefore, also on the basis of this functional cellular evidence, we have estimated of the utmost importance to carry out a more quantitative kinetic analysis of this process by MMP-2 on collagen type IV from human placenta at 37°C and at pH 7.3, comparing it with the behaviour of the catalytic domain of MMP-2, in order to characterize also the role of the hemopexin-

like domain in the recognition and cleavage of collagen IV. The experiments have been carried out under non denaturing conditions (since the temperature of 37°C is not denaturing collagen IV triple-helix, but only loosening it, see ref. Tanjore et al., 2001),even though the electrophoretic analysis is undertaken under denaturing conditions, so as to follow the degradation of individual polypeptides. We focussed our observations on three types of polypeptides, namely those corresponding to a molecular mass of 207, 169 and 92 kDa in collagen type IV from human placenta. On the basis of sequence based mass spectrometry determination in collaboration with Overall Lab, University of Vancouver, Canada (Himelstei et al., 1994; Hostikka and Tryggrason, 1987) the first two polypeptides are corresponding to the whole α-1 chain and to a fragment of the α-1 chain, lacking the 7S domain at the amino terminal of the polypeptyde. The third polypeptide is probably a peptide of α-2 chain which lacks of the NC1 domain at the C-terminal of the polypeptide sequence, resulting from the extraction treatment by pepsin, as suggested by others (Sundaramoorthy et al., 2002).

The results obtained (see Figs. 1 and 2 and Table 1) suggest that the lack of the 7S domain doesn't affect the catalytic efficiency of MMP-2 on the α1 chain of collagen IV. Therefore, if we compare the catalytic parameters for the processing of the α-1 and α-1 fragment (characterized by 207 and 169 kDa, respectively) by whole MMP-2 a closely similar behaviour is observed not only for the catalytic efficiency (as from k_{cat}/K_m, see Table 1), but also (within a factor of two) for the recognition mechanism and the rate-limiting cleavage event (i.e., K_m and k_{cat}, see Table 1), clearly suggesting a very similar proteolytic process by MMP-2 on the two polypeptides. However, MMP-2 displays a much reduced catalytic efficiency on the polypeptide, characterized by a molecular mass of 92 kDa, which correspond to the triple helical part of the α2 chain (as from Table 1). This feature seems mostly due to a reduced affinity (as from the higher K_m) of MMP-2 for this fragment wich respect to the other polypeptides of the whole α-1 chain and to the α-1 chain lacking the 7S domain; this reduced affinity is only partially compensated by a much faster rate-limiting step kinetic constant.
These different features are maintained also for the catalytic domain of MMP-2, thus suggesting they are not related to the presence of the hemopexin-like domain (see Table 1).

We have also carried out an investigation of the proteolytic processing by whole MMP-2 of native collagen type IV from murine Engelbreth-Holm-Swarm (EHS) sarcoma, which has been reported to be partially cleaved at 37°C by gelatinase A (Mackay et al., 1990; Eble et al., 1996).

In this case, we have followed the enzymatic processing of fragments corresponding to 225, 195 and 169 kDa, respectively, which appear to be the only ones to be processed by MMP-2. It is very likely that these fragments refer to three types of chains, one of which (i.e., the 169 kDa fragment) has the same molecular mass of the α-1 chain fragment and the 195 kDa fragment has a molecular mass very close to that reported for the whole α-1 chain. (Himelsten et al., 1994: Hostikka and Tryggrason 1987).Catalytic parameters for the proteolytic cleavage of these three fragments by whole MMP-2 are reported in Table 2. It comes out very clear that the enzymatic efficiency is drastically lower by about three orders of magnitude than for the collagen type IV from human placenta (see Table 1), especially if we compare the fragment which seems identical in the two types of collagen IV (i.e., the 169 kDa fragment). A closer comparison for the catalytic parameters of this fragment indicates that the lower proteolytic efficiency is mostly due to a very slow rate constant for the cleavage rate-limiting step, which is about 100-fold lower, and also in part to a tenfold decrease for the substrate affinity (as from the higher K_m, see Tables 1 and 2).

In order to have a deeper information on the determinants of the recognition process, we have compared the fragmentation of collagen type IV from human placenta by whole MMP-2 in the presence and in the absence of the rCBD domain, which has been reported to simulate the interaction of the fibronectin-like domain of MMP-2 with both collagen I and collagen IV (Tam et al., 2004; Xu et al., 2001 ; Steffensen et al., 2002). The results clearly indicate a marked inhibition of the proteolytic processing (see Fig. 5) by rCBD, suggesting that indeed whole MMP-2 interacts with the chains of collagen type IV from human placenta through its fibronectin-like domain, as showed for collagen I (Tam et al., 2004).

As a whole, from these data it comes out that the complexity of the structural assembly of collagen type IV indeed represents a limitation for the proteolytic action of MMP-2 (and likely of other MMPs), but this seems especially true for the cleavage itself of the chains (which is dramatically slowed down in the native form from murine EHS sarcoma with respect to that from human placenta, see Tables 1 and 2), whereas the recognition process for the formation of the ES complex does not appear particularly compromised by the tight network formed by α1 chain and its fragment. This feature, turns out to be true mainly for the α-1 and for its fragment, while the catalytic efficiency and the affinity of MMP-2 is very low for the triple helical part of the α2 chain.

However, it must be pointed out the enzymatic processing of collagen IV by MMP-2 has twofold contradictory effect.
Thus, while on one side the action of MMP-2 on the chains of collagen IV allows the exposure of cryptic sites, likely enhancing the susceptibility to the enzymatic processing by other protease (Koiuvnen et al., 199), on the other and gelatinase A seems also responsible for a negative effect on neutrophil migration thanksto the production of anti-angiogenic fragmentation products (see Fig. 4). This observation clearly indicates the complexity of effects associated to the activity of MMP-2 (and probably of other MMPs), arising the question whether a simple inhibitory effect on this activity can be advantageous for the control of pathological effects associated to it.
It is well known that Gelatinase B (MMP-9) is a matrix metalloproteinase, heavily expressed in neutrophils (Van den Steen et al., 2002), which is involved in the "angiogenesis switch" (Bergers et al., 2000) and in cancer progression (Himelstein et al., 1994; Itoh et al., 1998), as well as in vascular invasion (Ueda et al., 1996) and in chronic inflammatory processes associated to multiple sclerosis (Van den Steen et al., 2002). Therefore, since collagen IV is the main component of the basement membrane (Timpl et al., 1981; Kühn et al., 1981; Kühn, 1994; Kalluri, 2003; Tanjore and Kalluri, 2006), the molecular mechanism of its enzymatic processing by MMP-2 is of great pathophysiological significance. Therefore we have decided to investigate (i) the enzymatic processing of type IV of collagen also by MMP-9 and on (ii) the functional interrelation between the enzymatic action of the two gelatinases MMP-2 and MMP-9. We have analyzed in a quantitative fashion the enzymatic mechanism of MMP-9 concerning the first cleavage step of collagen type IV, which follows the Michaelis-Menten scheme. Data reported in Table 3 report the catalytic parameters for the first cleavage step, involving the same type of polypeptides for MMP-9 and MMP-2. It is important to notice that the catalytic efficiency of MMP-9 (as expressed by k_{cat}/K_m) is much lower for MMP-9, as compared to that of MMP-2 (Monaco et al., 2006), and this can wholly be attributed to the much lower affinity of MMP-9 for collagen IV (as expressed by K_m, see Table 3 and Monaco et al., 2006). It is evident that, unlike MMP-2 (Whicfh shows closely similar catalytic paraeters for the whole α1 and the α1 fragment without the 7S domain, see Table 1) α-1 chain fragment is more easily enzymatically processed by MMP-9 than the whole α-1 chains (as from values of k_{cat}/K_m, see Table 3). A feature which is completely referable to a much higher affinity (as from values of K_m, see Table 3) for the α-1 fragments. This indicates that the 7S portion seems to play a role for the

interaction with MMP-9, partially impairing its binding to the whole α1 chain (increasing Km, see Table 3), while it does not appear to affect the interaction of MMP-2 with thw whole α1 chain (see Table 1). This feature envisages the possibility that MMP-2 and MMP-9 interact at topologically different positions of the whole α1 chain. Further, the posibility of a topologically distinct binding site for MMP-2 is also supported by the evidence, that unlike MMP-2, the affinity of MMP-9 is much higher (by about 10-fold) for the native collagen IV from murine EHS sarcoma than for the pepsinized one from human placenta and this in spite of the much slower enzymatic activity on the native collagen IV (see Table 3), likely because of a much more compact network assembly and a different binding site. On the other hand, α-1 chains appear more susceptible to be cleaved than their fragments (as from k_{cat}, see Table 3), possibly because of the lack of the 7S domain that increases the speed rate-limiting cleavage event of MMP-9 (see k_{cat} value, Table 3).

The existence of two different binding sites for MMP-2 and MMP-9 is also strengthened by the synergistic action exerted by the Collagen Binding Domain from MMP-2 (CBD) on the enzymatic action of MMP-9 (see Fig. 8A and Table 4). Thus, it is evident that the enzymatic processing of both chains of type IV collagen from human placenta is greatly accelerated in the presence of CBD, underlying the occurrence of a ligand-linked conformational change of the collagen IV network upon binding of CBD, which facilitates the proteolytic attack by MMP-9. We have then carried out a thourough analysis of this effect (which appears to be present also for the native collagen IV from murine EHS sarcoma, see Fig. 9) as a function of CBD concentration (see Fig. 8A). It is evident that the catalytic efficiency (as from k_{cat}/K_m, see Fig. 8B) displays a bell-shaped dependence, suggesting the existence of two binding sites for CBD on all chains; this behaviour is present for all three fragments we have been investigating, but it looks especially marked for the triple helical part of the α-2 chain. In particular, Fig. 8A shows that the first binding site, which acts synergistically with MMP-9, is characterized by a higher affinity, whereas at higher concentrations CBD binds a second site, which competes with MMP-9, bringing about a decrease of the overall catalytic activity. These features indicate that the high affinity binding site for CBD is topologically distinct from that of MMP-9, and binding of CBD to this site induces a conformational change of the chain(s), which renders it more susceptible of the proteolytic attack by MMP-9; on the other hand, the second binding site,

characterized by a lower affinity, has an inhibitory effect on MMP-9 activity, likely because it overlaps with the binding site for MMP-9.

Therefore, the minimum scheme for describing this behaviour (see Scheme I) is to postulate the existence of one noncompetitive allosteric site (characterized for CBD by $^{1}K_{bu}$ in the absence of MMP-9 and by $^{1}K_{bL}$ in the presence of bound MMP-9) and a second competitive inhibitory site (characterized for CBD by $^{2}K_{bu}$). These parameters, which can be obtained by the analysis of data reported in Fig. 8 according to Eqs. 8a, 8b and 8c, are reported in Table 4 and they allow a quantitative description of the whole phenomenon. It emerges that

i) the ligand-linked effect on the affinity of CBD for the first binding site (i.e., $^{1}K_{bL}/^{1}K_{bu}$) is very limited for the α-1 (i.e., 207 kDa band) and α-1 chain fragment (i.e., 169 kDa band), being much less than a twofold affinity increase, whereas it is much larger for the triple helical part of α-2 chain (where a forty-fold affinity increase is observed, see Table 4);

ii) the ligand-linked effect related to the interaction of CBD with the first binding site is more pronounced on k_{cat}/K_m (i.e., $(^{1}k_{cat}/K_m)/(^{0}k_{cat}/K_m)$) for α-1 chain (with a twofold increase of k_{cat} and an almost twofold decrease of K_m, see Table 4), almost undetectable for the α-1 chain fragment and dramatic for the triple helical part of α-2 chain (with a threefold increase of k_{cat} and a thirty-fold decrease for K_m, see Table 4);

iii) the second competitive binding site for CBD is characterized by a much lower affinity with respect to the first binding site for the α-1 chain and the α-1 chain fragment, but it shows an almost two hundred-fold enhancement in the case of the triple helical part of α-2 chain (see Table 4).

In this respect, it may be important to outline that the increase of catalytic efficiency, induced by CBD, is much lesser than what observed for the denaturation of collagen type IV (see Table 3), suggesting that the CBD-induced conformational change could be only a partial unwinding of the polypeptide, much less extendend than in the case of denaturation. As a whole, a drastically different behaviour exists between the α-1 chain and the α-1 chain fragment of collagen IV one side and the triple helical part of α-2 chain on the other side. Thus, the CBD binding effect is much more reduced, though clearly evident, for the α-1 chain and α-1 chain fragment, envisaging a small but meaningful conformational transition in this assembled triple

helical structure; on the other hand, the CBD induced effect is dramatic in the case of the 92 kDa, underlying the possibility that when CBD binds the coiled structure, the conformation possesses a more efficient binding pocket for MMP-9.This interpretation finds a functional support on the fact that the triple helical part of α-2 chain displays an affinity constant for CBD much smaller than the α-1 chain and the α-1 chain fragment (see $^{1}K_{bu}$ in Table 4), but it acquires a similar affinity upon MMP-9 binding (see $^{1}K_{bL}$ in Table 4) and the same occurs for K_m of MMP-9 upon CBD binding (see $^{0}K_m/^{1}K_m$ in Table 4).

Therefore, this potentially relevant interaction between the two binding sites for MMP-2 (represented by the first allosteric site for CBD binding) and that for MMP-9 (represented by the competitive site for CBD) may represent an important clue of the mechanism operative "in vivo" on collagen IV, as indicated by the fact that the same behaviour is observed on native collagen IV from murine EHS sarcoma (see Fig. 9). It is interesting to observe that this behaviour is drastically different from that reported for collagen I, where it has been suggested that the binding sites for the two gelatinases are overlapping (Xu et al., 2005). This phenomenon indeed suggests that in the case of collagen IV the two gelatinases can cooperate "in vivo" both catalytically (if they proteolytically attack together the collagen IV at different binding and cleavage sites) but also allosterically in the absence of the enzymatic activity of either one of the two gelatinases, since, as shown in this paper, even the inactive CBD is able to cooperate with MMP-9 in processing collagen IV.

The synergistic effect between MMP-2 and MMP-9 on collagen IV cleavage, has been tested also *ex vivo*, through an experiment of neutrophil cells migration across a collagen IV coated membrane. Neutrophil cells are able to express different kind of proteolytic enzymes, such as MMP-8, MMP-9 and neutrophil elastase, while they don't produce a valuable amount of MMP-2.

So, it has been possible to test the activity of endogenous MMP-9 on type IV collagen in the presence of different forms of exogenous MMP-2. MMP-9, in the presence of CBD, and in the presence of different biochemical forms of MMP-2 (pro-MMP-2 *column 6* and MMP-2 without the catalytic domain *column 5*), cleaves collagen IV with an higher catalytic efficiency.

We can conclude that both MMP-2 and MMP-9 process collagen type IV, the main component of BM, in order to allow the proliferation of cells across basement membrane during physiological and pathologial processes probably with a synergistic effect that increases their proteolytic activity.

Conclusion

Proteolytic degradation of basement membrane influences the cell behaviour during important processes, such as inflammations, tumorigenesis, angiogenesis and allergic diseases.
Angiogenesis is an important biological process considered fundamental for reproduction, development and repair. In the adult, repair and reproductive angiogenesis occur mainly as brief bursts of capillary blood-vessel growth that usually lasts only days or weeks. This physiological angiogenesis is tightly regulated by a variety of circulating or sequestred inhibitors that suppress proliferation of vascular endothelium.
Type IV collagen is the main component of the VBM, and its degradation serves multiple purpose, which include the liberation of endothelial cells to migrate and proliferate from their cell-surface integrins, the liberation of sequestred growth factors (VEGF, βFGF) and the detachment of the pericytes that surround and support the blood vessels.
Gelatinases play a central role in type IV collagen degradation as well as in regulating proteolytic activities of several other constituents of the extracellular matrix. Gelatinase MMP-9 (Gelatinase B) and the other Gelatinase A (or MMP-2) possess a unique collagen binding domain (CBD), called fibronectin-like domain, inserted between the catalytic site and the Zn^{2+}-binding domain and consisting of three 58-amino acid fibronectin type II-like modules (Allan et al., 1995). Further, only in the case of MMP-9 the catalytic domain and the hemopexin domain are separated by a long O-glycosylated domain, which is considerably shorter or not present in other MMPs (Van den Steen et al., 2006).

MMP-2 and MMP-9, like other MMPs, are implicated in various physiological and pathological processes. The physiological role of gelatinases concerns mainly normal tissue homeostasis and extracellular matrix remodelling. The unbalanced degradation of substrates often results in pathological conditions (Van den Steen et al., 2002). Since MMP-9 and MMP-2 are involved in normal bone development (Vu et al., 1998), inappropriate control of their synthesis, excess secretion or activation may result in pathological bone resorption, such as in the morbus of Paget, amyloidosis, hyperparathyroidism, giant cell tumors and osteolytic metastases (Ohashi et al., 1996; Ueda et al., 1996; Vidovszky et al., 1998). In addition, an upregulation or activation of MMP-9 has been shown during different types of inflammatory and degenerative diseases, such as in rheumatoid arthritis (Masure et al., 1991; Van den Steen et al., 2002), multiple sclerosis (Opdenakker et al., 2003), Alzheimer's disease (Lorenzl et al., 2003; Adair et al., 2004), amyotrophic lateral sclerosis (ALS) (Lim et al.,

1996), atherosclerosis (Loftus et al., 2000; Gough et al., 2006) and pancreatitis (Descamps et al., 2003). Since MMP-2 is a rather constitutively expressed enzyme and MMP-9 is induced by many inflammatory stimuli, both enzymes may act together in the context of inflammation. Further, it has been suggested that both MMP-9 and MMP-2 are also present during tumor progression and metastasis (Itoh et al., 1998), since they are actively involved in the basement membrane proteolysis during cancer cell diffusion and invasion (Himelstein et al., 1994). This statement is in line with the observation that tumour invasivity is related to chemokine production, which, in turn, recruit gelatinase B from the host neutrophils (Opdenakker et al., 1992). This is a crucial element for tumour growth, since it stimulates angiogenesis, inducing the primary vessels, created during the embryonic stage, to differentiate and new blood vessels to sprout and to branch from pre-existing capillaries.
In particular, MMP-9 and MMP-2 seem to be involved in the induction of a new vasculature during tumour dissemination and wound healing, and their role has been demonstrated in endothelial cell morphogenesis leading to tube formation on Matrigel (Schnaper et al.,1993). Moreover, synthetic inhibitors of gelatinases A and B prevent tumor growth and invasion through an antiangiogenic targeting (Koivunen et al., 1999; Eccles et al., 1996).
Gelatinases (MMP-9 and MMP-2) degrade type IV collagen, probably distrupting the organization of VBM, and leading to the release of BM-bound VEGF. This release of VEGF leads to the angiogenic switch ; the endothelial cells begin to proliferate in a provisional matrix in order to form new blood vessels (Kalluri et al., 2003).
The degradation of VBM by gelatinases also leads to the generation of fragments with antiangiogenic activity, such as endostatin, arrestin, canstatin, tumstatin and other collagen fragment (Kalluri et al., 2003). MMP-mediated degradation of BM can therefore act as both a positive (early stage) and a negative (middle to late stage) regulator of tumour angiogenesis .

In this study, we have investigated the action of gelatinase A (MMP-2) and B (MMP-9) on collagen IV comparing quantitatively their actions on the soluble forms of collagen IV extracted with or without pepsin (from human placenta and from Engelbreth-Holm-Swarm (EHS) murine sarcoma, respectively). We have analyzed the capability of gelatinases to cooperate during collagen IV degradation. We have also studied the different role of MMP-2 domains in collagen IV degradation and its capability to produce collagen IV fragment in order to prevent neutrophil migration across a

collagen IV membrane. The catalytic efficiency of MMP-2, and also MMP-9, on type IV collagen from murine sarcoma (EHS) is dramatically reduced, with respect to that on the type IV of collagen from human placenta, probably because of the much tighter packing of the network which renders very slow the speed of the rate-limiting step. In the case of type IV collagen from human placenta, the catalytic efficiency (as expressed by k_{cat}/K_m) on the α-1 (i.e., 207 kDa) and the α-1 chain fragment (i.e., 169 kDa) is much lower for MMP-9, as compared to that of MMP-2 (Monaco et al., 2006), and this can wholly be attributed to the much lower affinity of MMP-9 for collagen IV (as expressed by K_m, see Table 1 and Monaco et al., 2006). This should reflect either a different interaction mode of the two enzymes (likely due to a different conformation for fibronectin-like domain in the two enzymes) and/or a topologically distinct binding site, as suggested by cooperative effect exerted by CBD on MMP-9 action (see Fig. 8A).

However, for both types of collagen IV the presence of the Collagen Binding Domain of MMP-2 (CBD) enhances the enzymatic processing by MMP-9. This effect clearly indicates that the fibronectin-like domain of MMP-2 and MMP-9 bind to topologically distinct sites on type IV collagen, bringing about a conformational change of the collagen IV molecule. This allows the two enzymes to cooperate with each other through a ligand-linked mechanism, which does not necessarily require the enzymatic action. Therefore, fibronectin-like domains not only increase the affinity between enzyme and substrate to enhance the catalysis, they also act as allosteric third party elements in the MMP action.

Moreover, we have also enquired on the role of MMP-2 domains in processing collagen IV. The catalytic domain of MMP-2 has only a limited effect on the catalytic efficiency toward collagen IV, indicating that the lack of the hemopexin like domain has not a great relevance for the overall mechanism. Instead, the addition of the isolated collagen binding domain, corresponding to the fibronectin-like domain of whole MMP-2, greatly inhibits the cleavage process, demonstrating that MMP-2 interacts with collagen type IV preferentially through its fibronectin-like domain.

We have also investigated the activity of neutrophil cells to migrate across a membrane coated with both types of collagen IV, in a chemiotaxis assay, with and without the addition of (i) exogen whole MMP-2, (ii) MMP-2 without the catalytic domain, (iii) the collagen binding domain of MMP-2 or CBD and (iv) inactive MMP-2 or pro-MMP-2. It is important to underline that neutrophils extracts do not contain appreciable amounts of MMP-2 activity, as from the gelatin zymographic analysis (see inset in Fig. 4), but

only MMP-8 collagenase activity and MMP-9 gelatinase activity. Moreover, gelatinase activity is absolutely required for the neutrophil migration, because MMP-8 has not the capability to process type IV of collagen. The addition of exogen MMP-2 to cells solution brings about a decrease of neutrophils migration, probably due to a MMP-2-induced formation of fragments of type IV of collagen with anti angiogenic activity. On the other hand, the addition of both MMP-2 without the catalytic domain and CBD and pro-MMP-2 increases the activity of endogenous neutrophil MMP-9 on collagen IV.

In conclusion, both gelatinases can process type IV of collagen and can cooperate “in vivo” both catalytically (if they proteolytically attack together the collagen IV at different binding and cleavage sites) but also allosterically in the absence of the enzymatic activity of either one of the two gelatinases, since, as shown in this paper, even the inactive CBD is able to cooperate with MMP-9 in processing collagen IV.

Materials and Methods

I) Materials

Type IV collagen from human placenta (Sigma Chemical Co. S t. Louis, MO, USA) was dissolved in acetic acid 0.1 M at room temperature to a final concentration of 1 mg/ml. The suspension was centrifuged for 1 hr at 10,000 x g and the supernatant, containing the dissolved collagen, was used. The final amount of solubilized substrate has been quantified as described by Bradford [29].

Native type IV collagen from Engelbreth-Holm-Swarm murine sarcoma (EHS) (Sigma Chemical Co. St. Louis, MO, USA) was dissolved in acetic acid 0.1 M at room temperature to a final concentration of 0.75 mg/ml. The suspension was centrifuged for 1 hr at 10,000 x g and the supernatant, containing the dissolved collagen, was used. The final amount of solubilized substrate has been quantified as described by Bradford [29]. For the experiment of MMP-9 digestion of denatured collagen IV from human placenta, collagen IV was dissolved in 0.1 M acetic acid at room temperature to a final concentration of 1 mg/ml. The suspension was boiled for ten minutes to allow protein denaturation and then centrifuged for 1 hr at 10,000 x g. The supernatant, containing the dissolved collagen, was used immediately for fragmentation experiments. 8 µg of collagen IV from human placenta (Sigma Chemical Co. St. Louis, MO, USA) was separated by reducing SDS-PAGE and blotted onto a PVDF membrane. The membrane was stained with Coomassie Brilliant Blue R250 and destained. The 92 kDa band was excised and sequenced on a capillary protein sequencer (Procise 491 cLC; Applied Biosystems, Foster City, CA, USA).

MMP-2 recombinant proenzyme was either of commercial origin (R&D System, London, UK) or a generous gift of Dr. Chris Overall (Univ. of British Columbia, Canada); no significant functional difference has been detected between the two preparations after activation. The catalytic domain (cdMMP-2) of human recombinant MMP-2 (Biomol International, USA) was dissolved in a solution of 50 mM Tris/HCl, 0.1 M NaCl, 10 mM $CaCl_2$ at pH 7.2.

The recombinant fibronectin-like domain (rCBD) of MMP-2 was a generous gift of Dr. Chris Overall (Univ. of British Columbia, Canada) and its identity was verified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (30) after running a small amount of rCBD on a small column of gelatin Sepharose (Sigma Chemical Co. St. Louis, MO, USA) [31].

Recombinant human full length MMP-9 was produced in Sf9 insect cells after transfection with a baculovirus carrying the MMP-9 cDNA (3). The secreted proMMP-9 was purified to homogeneity by gelatin-Sepharose chromatography (9) and dialyzed into 100 mM Tris/HCl pH 7.4, 100 mM NaCl, 10 mM $CaCl_2$. The pro-enzyme was activated by incubating 10 μM progelatinase solution with a solution of 0.1 μM cdMMP-3 (Biomol International, USA) at 37°C for 90 minutes.
The quenched fluorogenic substrate MCA-Pro-Leu-Gly-Leu-DPA-Ala-Arg-NH_2 was purchased from Calbiochem (La Jolla, CA, USA).
Lipopolysaccharide W E. Coli LPS was purchased from DIFCO Laboratories (Detroit Michigan, USA). Orthophenilendiamine (OPD) was purchased from Sigma Chemical Co. St. Louis, MO, USA). GM6001 (Ilomastat) was purchased from BioMol Int. (Hamburg, Germany).

II) Methods

1) Enzymatic Activation

Isolated purified MMP-2 proenzyme was activated by incubating 0.1 ml of a 0.1 μg/ml progelatinase solution with p-aminophenyl mercuric acid at 37°C for an alf hour. The human recombinat full length MMp-9 pro-enzyme was activated by incubating 10 μM progelatinase solution with a solution of 0.1 μM cdMMP-3 (Biomol International, USA) at 37°C for 90 minutes.

2) Activity Assay

The active amount of the enzyme MMP-2 and MMP-9 were determined by gelatin zymography and by the fluorimetric assay, as described by others (Knight, et al 1992) [32], following the progressive decrease of hydrolysis of the quenched fluorogenic substrate MCA-Pro-Leu-Gly-Leu-DPA-Ala-Arg-NH_2(λ_{exc} = 325 nm, λ_{em} = 393 nm) upon addition of Batimastat (BB-94), a peptidomimetic inhibitor (kindly provided by British Biotech Pharmaceutical, Cowley, Oxford, UK), which stoichiometrically inhibits MMPs.
All measurements were performed at 37°C using a solution of 50 mM Tris/HCl, 0.1 M NaCl, 10 mM $CaCl_2$ plus 0.05% Brij 35 buffered at pH 7.3.

3) Kinetics of digestion of collagen type IV from human placenta and of native collagen type from murine EHS sarcoma by whole MMP-2 and cdMMP-2

For substrate fragmentation kinetics, activated whole MMP-2 was added to collagen type IV solutions from human placenta/or from murine EHS sarcoma, at a final concentration of 10 pM, while the catalytic domain of MMP-2 was added to collagen solutions at a final concentration of 15-20 pM. The kinetics has been carried out in 50 mM Tris/HCl, 0.1 M NaCl, 10 mM $CaCl_2$ at pH 7.2, employing different concentrations of collagen type IV (spanning between 1 μM and 4 μM). Kinetics has been carried out keeping the mixtures at 37°C and harvesting small aliquots at different time intervals. Reactions were stopped by the addition of SDS-PAGE loading buffer containing 20 mM EDTA and frozen to – 80°C until used.

The aliquots in reducing sample buffer were separated on a 4-15% gradient SDS-PAGE gels**,** which were stained using 0.5% Coomassie Blue and destained in 10% acetic acid and 40% methanol until substrate bands were clearly visible or employing a Silver Staining solution. The broad spectrum protein markers (Bio Rad, USA) were used as molecular weight standards.

4) Human neutrophils isolation

Neutrophils were isolated from healthy donors volunteers. Whole blood was diluted (blood-PBS1x ratio 1:4) and stratified on Ficoll separating solution, then centrifugated at 1400 rpm for 30 min. at 4 °C. Plasma and mononuclear cells were then removed by aspiration and the red blood cells lysed by adding 18ml of ice-cold water for 30 sec.; thereafter 2 ml of PBS 10x were added and suspension was centrifuged (1,600 rpm for 5 min. at 4°C) in order to remove RBC membranes debris and haemoglobin.

The neutrophils containing pellet was then washed twice at 1600 rpm for 5 min. at 4°C and resuspended in RPMI 1640 (EuroClone) medium supplemented with BSA (0,2%) to a final concentration of $4x10^6$ cells/ml.

Viability of purified cells was measured by Tripan blue dye exclusion count and it was found always >95%.

5) Chemotaxis assay

Human neutrophils chemotaxis assay was measured in Falcon 24-well plates containing transwell inserts with 3.0 μm pore diameter [33]. Transwell inserts were coated overnight with type IV collagen from human placenta (Sigma Chemical Co. St. Louis, MO, USA) or from murine EHS sarcoma previously dissolved in acetic acid 0.1 M at room temperature and diluted with PBS 1x to a final concentration of 10 μg/ml.

Lower chambers contained 500 ml of RPMI 1640 medium supplemented with 0,5% BSA, to which we have added (for different experiments) :

- LPS to a final concentration of 500 ng/ml as a positive control;
- LPS 500 ng/ml and exogenous MMP-2 at different concentrations spanning between 1 and 40 nM;
- LPS 500 ng/ml, exogenous MMP-2 to a final concentration of 1 nM and Ilomastat (a powerful inhibitor of MMPs, see ref. [34]) to a final concentration of 100 μM**;**
- LPS 500 ng/ml and the digestion product (250 μl) of collagen IV by 5 nM MMP-2. In this experiment, type IV collagen (20 μg/ml) was digested for 6 hours with MMP-2 to obtain 50% degradation measured in SDS-PAGE; after degradation, MMP-2 was stoichiometrically inhibited with Ilomastat, a synthetic MMPs inhibitor, as confirmed by fluorimetric assay. This experiment was performed on uncoated transwell in order to directly measure the effect of type IV collagen digested fragments on MMP-independent LPS-mediated chemiotaxis.

In the upper compartment 250 μL of neutrophils (10^6 cells/well) have been added.

Cells were allowed to transmigrate for 4 h at 37 °C in a humidified 5% CO_2 incubator .

To evaluate the transmigrated neutrophils, the Transwell inserts were removed after washing the lower part of membrane and the bottom solution was collected and centrifuged; cell pellets were then suspended in a solution containing Triton X-100 to a final concentration of 0.5%, OPD 600 μg/ml and H_2O_2 6 μl/ml in PBS 1x.

Diluted concentrations of neutrophils, from 10^6 cells/well to $6x10^4$ cells/well were used to obtain a standard curve.

The oxidation of OPD performed by myeloperoxidase contained in neutrophils was stopped by adding 40 μL of HCl 2M; the reading has been performed at 492 nm in a spectrophotomer (Jasco V-530). Any experiment

was performed in triplicate at least with two different blood samples from different donors.

6) Role of the fibronectin-like domain of MMP-2 (rCBD) on the processing of collagen type IV from human placenta by whole MMP-2

The fibronectin-like rCBD domain of MMP-2 (at a final concentration of 50 μM) has been incubated with collagen type IV from human placenta (1mg/ml) for 30 minutes at 37°C to allow the interaction to occur. After this time period, MMP-2 was added to the mixture and the solution was put for other 30 minutes at 37°C. At the same time, three additional control solutions have been prepared, namely i) collagen type IV alone, ii) collagen type IV incubated for the same time with MMP-2 at 37°C, and iii) a solution of collagen type IV with 50 μM rCBD, but without MMP-2.

Reactions were stopped by the addition of SDS-PAGE loading buffer containing 20 mM EDTA and frozen to – 80°C until used. The different samples were visualized by SDS-PAGE electrophoresis.

7) Kinetic Analysis

Electrophoretic spots, corresponding to different aliquots at different time intervals, have been analysed by a laser densitometer (LKB 2202 UltraScan) and their intensity has been calibrated (in order to obtain concentration values) using standard substrate solutions. For the different species the substrate disappearance rates were derived at each concentration of type IV collagen employed.

The measurement of the initial velocity has been referring to a time period of one hour for collagen type IV from human placenta and three hours from native collagen type IV over which less than 10% of the substrate was degraded during the assay, and in any case the analysis was limited to the time interval over which linearity of the rate was observed. It ensured a steady-state condition for the first cleavage step, and it was a prerequisite for the subsequent analysis step. It consisted of the verification for the applicability of the Michaelis-Menten approximation to the first cleavage step, which was based on the observation of an inverse linear correlation between velocity and substrate concentration according to the Lineweaver-Burk equation to obtain the catalytic parameters k_{cat} and K_m

$$\frac{E_0}{v} = \frac{K_m}{k_{cat}} \cdot \frac{1}{[S]} + \frac{1}{k_{cat}}$$

(Equation 1)

where E_0 is the total enzyme concentration, v is the actual rate (expressed as mol/s), K_m is the Michaelis-Menten equilibrium constant (expressed as mol), k_{cat} is the rate-limiting step kinetic constant (expressed as s^{-1}) and [S] is the substrate concentration.

8) Kinetics of digestion of collagen type IV from human placenta and of native collagen type IV from murine EHS sarcoma by MMP-9

For substrate fragmentation kinetics, activated intact MMP-9 was added to collagen type IV solutions, at a final concentration of 30 pM. Kinetic analyses were carried out in 50 mM Tris/HCl, 0.1 M NaCl, 10 mM $CaCl_2$ at pH 7.1, employing different concentrations of collagen type IV (spanning between 1 μM and 3 μM). Kinetics were carried out keeping the mixtures at 37°C and harvesting small aliquots at different time intervals. Reactions were stopped by the addition of SDS-PAGE loading buffer containing 20 mM EDTA and frozen to – 80°C until used.

The aliquots in reducing sample buffer were separated on a 4-15% gradient SDS-PAGE gels**,** which were stained either using 0.5% Coomassie Blue and destained in 10% acetic acid and 40% methanol until substrate bands were clearly visible or with Silver Staining. The broad and low spectrum protein markers (Bio Rad, USA) were used as molecular weight standards.

9) Contribution of the fibronectin-like domain of MMP-2 (CBD) on the processing of collagen type IV from human placenta by MMP-9

Different concentrations of the fibronectin-like domain of MMP-2 (CBD) (spanning between a final concentration of 1 μM and 150 μM) were incubated with collagen type IV from human placenta (1mg/ml) for 30 minutes at 37°C to allow the interaction to occur. After this period, MMP-9 was added to the mixture (to a final concentration of 30 pM) and the solution was incubated for other 30 minutes at 37°C. At the same time, three additional control solutions were prepared, namely i) collagen type IV alone,

ii) collagen type IV incubated for the same time interval with MMP-9 at 37°C, and iii) a solution of collagen type IV with the respective concentration of CBD, but without MMP-9.

Reactions were stopped by the addition of SDS-PAGE loading buffer containing 20 mM EDTA and frozen to – 80°C until used. The different proteins were visualized after SDS-PAGE electrophoresis.

10) Contribution of the fibronectin-like domain of MMP-2 (CBD) on the processing of native collagen type IV (EHS) by MMP-9

The fibronectin-like domain of MMP-2 (CBD) (at a final concentration of 25 μM) has been incubated with native collagen type IV (0.75 mg/ml) for 30 minutes at 37°C to allow the interaction to occur. After this period, MMP-9 was added to the mixture and the solution was kept for 1 hour at 37°C. At the same time, three additional control solutions were prepared, namely i) collagen type IV alone, ii) collagen type IV incubated for the same time with MMP-9 at 37°C, and iii) a solution of collagen type IV with 25 μM CBD, but without MMP-9.

Reactions were stopped by the addition of SDS-PAGE loading buffer containing 20 mM EDTA and frozen to – 80°C until used. The different proteins were visualized after SDS-PAGE electrophoresis.

11) Kinetic Analysis

Electrophoretic spots, corresponding to different aliquots at different time intervals, were analysed by a laser densitometer (LKB 2202 UltraScan) and their intensity was calibrated (in order to obtain concentration values) using standard substrate solutions. It must be pointed out that different preparations of native type IV collagen or type IV collagen from human placenta displayed differing ratios of the relative amount of the species, as from the intensity of electrophoretic spots on SDS-PAGE under reducing conditions. For the different species the substrate disappearance rates were derived at each type IV collagen concentration employed.

The measurement of the initial velocity was referred to a time period of one hour for type IV collagen from human placenta and three hours from native type IV collagen. During these intervals, less than 10% of the substrate was degraded in the assay, and in any case the analysis was limited to the time interval over which linearity of the rate was observed. This ensured a steady-

state condition for the first cleavage step, and it was a prerequisite for the subsequent analysis step. This consisted of the verification for the applicability of the Michaelis-Menten approximation to the first cleavage step, which was based on the observation of an inverse linear correlation between velocity and substrate concentration according to the Lineweaver-Burk equation. In order to obtain the catalytic parameters k_{cat} and K_m data were simultaneously fitted to the Lineweaver-Burk equation

$$\frac{E_0}{v} = \frac{K_m}{k_{cat}} \cdot \frac{1}{[S]} + \frac{1}{k_{cat}}$$

(Equation 1)

and to the Eadie-Hofstee equation

$$\frac{v}{E_0} = k_{cat} - \frac{v}{E_0 \cdot [S]} \cdot K_m$$

(Equation 2)

where E_0 is the total enzyme concentration, v is the actual rate (expressed as mol/s), K_m is the Michaelis-Menten equilibrium constant (expressed as mol), k_{cat} is the rate-limiting step kinetic constant (expressed as s^{-1}) and [S] is the substrate concentration.

Abbreviations

BM: basement membrane
VBM: vascular basement membrane
ECM: Extracellular Matrix;
MMP: Matrix MetalloProteinases;
TIMP: Tissue Inhibitors of MetalloProteinases;
MT-MMP: Membrane Type MMPs;
LHD: linker/hemopexin-like domain;
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis;
ADAM: a disintegrin MMP;
NO: nitrix oxide;
FACIT: Fibril-Associated Collagens with Interrupted Triple helices;
NMR: Nuclear Magnetic Resonance;
APMA:4-aminophenylmercuric acetate;
CD: Circular Dichroism;
rCBD Recombinat collagen binding domain or fibronectin like domain

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Biochemical biologist/Molecular biologist
Strong oral and written communication skills –
Strong interpersonal skills
Adaptability - Challenging - Fast learning

Research Experience

Current research: Structural and functional analysis of Matrix Metalloproteases (MMPs) on natural substates. I have started an investigation on the enzymatic properties of MMP-2 and MMP-9 in order to better understand the interaction mode of these two proteins during pathological process such as inflammation and tumor progression. 2004/2007
research project: Investigation of the functional enzymatic properties of Gelatinases. In particular, the characterization of enzymatic mechanisms of gealtinase MMP-2 and MMP-9 in the proteolytic degradation of basement membrane components.
2003/2004 One-year research project: Discovering MMP-2 substrates and mechanistically dissecting its function and regulation by proteolysis in vivo was a major focus of the research. In particular the project was on the molecular basis of fibrinogen binding and cleavage by MMP-2. I have devoted my efforts toward the comprehension of the role of different domain in the modulation of the catalytic activity on fibrinogen.

Technical knowledge

Biochemistry	Western Blotting, Zymography, Protein purification. In-vitro protein production, Co-immuno precipitation, Protein assays. Enzymatic assay. Circular Dichroism technique.
Molecular Biology	DNA and RNA extractions, PCR, RT-PCR.

Cellular Biology	Cell cultures, (Co-)-Transfections, Transformations, cellular migration.

Academic Qualifications

2004	*PhD in Molecular biology and Biochemistry University of Rome "Tor Vergata" (It)*
	Laboratory of Biochemistry, Department of Experimental Medicine, Rome (It), Prof Massimiliano Coletta.
2004	*Italian professional abilitation to biological public work University of "Tuscia" Viterbo (It).*
2003	*Master's degree in Biology University of Rome "Roma Tre" (It).*
	Biochemical thesis, Prof. Paolo Ascenzi.
1998	*High school license At Scientific Liceum "J.F. Kennedy" Rome (It).*

Teaching Skill

2003-up to date: Assistant teacher at Chemistry and propaedeutical Biochemistry course for the first year students of the Faculty of Medicine of University of "Tor Vergata" (It).
2004-up to date: Seminars for the Molecular Biology II course of the fourth years students of Biological Science of University of "Camerino". Camerino (It). "New factors involved in angiogenesis".

PhD Schools

2004:

Fast kinetic course. Winter school of the group of Enzymology and Metabolic Regulation of SIB (Società Italiana di Biochimica e Biologia Molecolare) Italy. Varese 16-20 Feb 2004.

Skills acquired

Time management, Organisation, Respecting tight deadlines, working under pressure.

Original analytical thinking, perspicacity, multi-task and multidisciplinary abilities

Innovative at problem solving and conceptualisation

Autonomy, working independently and as part of a team

Languages and other skills

Languages competent writing and speaking English and French .

IT Proficient in the use of the Internet, PCs and MACs computer programs. Microsoft Office, Image treatment, matlab user, Computer-assisted structural analysis.

Publications

Refereed publications:

Rodriguez J, Di Pierro D, Gioia M, **Monaco S**, Delgado R, Coletta M, Marini S. Effects of a natural extract from Mangifera indica L, and its

active compound, mangiferin, on energy state and lipid peroxidation of red blood cells. *Biochim Biophys Acta.* ***2006;1760(9):1333-42.***

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- Monaco S, **Gioia M**, Rodriguez J, Fasciglione GF, Lupidi G, Marini S, Coletta M *Proteolytic Activity of Matrix Metalloproteinase-2 (Gelatinase A) on Fibrinogen. Italian Biological Chemistry* 11-13 Nov 2004
- Gioia M., **Monaco S**., Fasciglione G.F., Marini S., Politi V., Coletta M. *Functional modulation of matrix metalloproteinase*. 2nd Workshop on Pharmaco-Bio-Metallics Certosa di Pontignano- Siena November29th - December1st 2002.

- **Monaco S.,** Gioia M., Fasciglione G.F. Marini S., Coletta M. *Differential aspects of gelatinase A (MMP-2) and gelatinase B (MMP-9) on type IV of collagen.*
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Oral presentations:

- Coletta M, **Monaco S**, Gioia M, Fasciglione GF, De Sanctis G, Marini S. *Modulation of the processing of natural substrates by gelatinose MMP-2 and MMP-14.* Second Chianti meeting on proteases Certosa di pontignano Siena Italy May 16-20, 2004.
- **Monaco S**, Fasciglione G.F., Di Pierro D., Marini S., Coletta M. *Differential aspects of gelatinases on type IV of collagen degradation.* Sesto Pharmaco Bio-Metallics. University of Napols "Federico II" 30/11-1/12, 2006.
- **Monaco S.,** Gioia M., Fasciglione G.F., Di Pierro D., Marini S., Coletta M. *Gelatinase A (MMP-2), Gelatinase B (MMP-9) and neutrophil elastase have distinct capability to process type IV of collagen.* 7nd Workshop on PharmacoBiometallics. 27th 28th October.

Privacy

Concerning the Italian privacy policy (Legge 675, 31-12-1996 and D.lgs. 196/2003), I hereby approve the use of the privacy protected content in this Curriculum Vitae.

Articles

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Effects of a natural extract from *Mangifera indica* L, and its active compound, mangiferin, on energy state and lipid peroxidation of red blood cells

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Abstract

Following oxidative stress, modifications of several biologically important macromolecules have been demonstrated. In this study we investigated the effect of a natural extract from *Mangifera indica L* (Vimang), its main ingredient mangiferin and epigallocatechin gallate (EGCG) on energy metabolism, energy state and malondialdehyde (MDA) production in a red blood cell system. Analysis of MDA, high energy phosphates and ascorbate was carried out by high performance liquid chromatography (HPLC). Under the experimental conditions, concentrations of MDA and ATP catabolites were affected in a dose-dependent way by H_2O_2. Incubation with Vimang (0.1, 1, 10, 50 and 100 μg/mL), mangiferin (1, 10, 100 μg/mL) and EGCG (0.01, 0.1, 1, 10 μM) significantly enhances erythrocyte resistance to H_2O_2-induced reactive oxygen species production. In particular, we demonstrate the protective activity of these compounds on ATP, GTP and total nucleotides (NT) depletion after H_2O_2-induced damage and a reduction of NAD and ADP, which both increase because of the energy consumption following H_2O_2 addition. Energy charge potential, decreased in H_2O_2-treated erythrocytes, was also restored in a dose-dependent way by these substances. Their protective effects might be related to the strong free radical scavenging ability described for polyphenols.

Keywords: Antioxidant; EGCG; *Mangifera indica* L; Mangiferin; Erythrocyte

1. Introduction

During electron transfer there is a production of free radicals, reactive oxygen species (ROS) and highly reactive oxygen-containing radicals by a wide range of different phenomena (e.g., oxidative metabolism of mitochondria, activation of inflammatory cells, hyperoxia, ischemia/reperfusion, the arachidonic acid cascade, cytochrome P450-mediated reactions and exposure to chemicals or physical agents, such as gamma rays) [1]. The first exhaustive experiments performed in human erythrocytes showed the ability of ROS to also induce lipid peroxidation and haemolysis [2]. Further experiments demonstrated that ROS are also responsible for a significant depletion of the pyridine coenzyme pool mainly because of ATP consumption due to the activity of reducing enzymes [3]. Both processes are responsible for a reduction of the energy charge (i.e., ATP and GTP concentrations) of most normal cells and for an increase of ADP cellular concentration. NAD/NADH ratio as well is raised due to the increased energy consumption while adenosine monophosphate (AMP) deaminase, one of the main targets of oxidative stress [3], seems to depress energy metabolism by blocking the rescue pathway of purine nucleotides.

Several types of cells, including erythrocytes, contain different defense mechanisms against free radical-induced lipid peroxidation, which include both enzymatic (such as

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catalase, superoxide dismutase and glutathione peroxidase) and non-enzymatic factors (e.g., reduced glutathione and other prominent representatives of dietary antioxidants, such as ascorbate (vitamin C), tocopherols (vitamin E), carotenoids, selenium and flavonoids). All these molecules have been shown to react with free radicals reducing reactive species-mediated damages both in vitro and in vivo [3]. Intracellular antioxidant species may act in vivo to decrease oxidative damage to DNA, protein and lipids, thus reducing the risk of systemic diseases complications and cancer [4,5].

Flavonoids are a large group of polyphenolic antioxidants that occur in several fruits, vegetables, and beverages such as tea and wine. Similarly, many plants contain a range of potential and powerful antioxidants. The biological, pharmacological and medical properties of polyphenols, have been also extensively reviewed [6,7].

Recently, a standard aqueous stem bark extract from selected species of *Mangifera indica* L. (Anacardiaceae), which has been used in pharmaceutical formulations in Cuba under the brand name of Vimang®, has been reported to show a potent in vivo and in vitro anti-inflammatory activity [8,9], immunomodulation on rat macrophages [10] and a strong in vitro and in vivo antioxidant effect [11,12].

Different studies demonstrated that mango stem bark extract could be useful to prevent the hyperproduction of reactive oxygen species (ROS) by peritoneal macrophages and from (TPA)-induced oxidative damage in serum, liver, and brain of mice, being more active than vitamin C, vitamin E, and β-carotene [12]. This extract also shows a very powerful scavenger activity for hydroxyl radicals ($OH^{\cdot}$) and hypochlorous acid, associated to a relevant inhibitory effect on the peroxidation of rat brain phospholipids and a protective effect against DNA damage induced by iron/bleomycin or copper-phenantroline models [11].

The chemical composition of Vimang ® has been characterized by chromatographic (planar, liquid and gas) methods, mass spectrometry and UV/Vis spectrophotometry [13]. The total polyphenol content of Vimang® determined by the Folin–Ciocalteu method and expressed as gallic acid equivalents, was 34 g/100 g dry weight. Chemical studies have enabled the isolation and identification of phenolic acids, phenolic esters, flavan-3-ols, mangiferin, which is the predominant component of this extract [13], and micronutrients as selenium [14].

The mangiferin content was determined by HPLC methods and found to represent about 13% of the powdered extract, i.e., about 50% of polyphenols with antioxidant activity present in crude mango extract. Mangiferin has been previously tested in vitro for its antioxidant [15–17], immuno-stimulating and antiviral properties [18] and it was found (i) to protect hepatocytes, lymphocytes, neutrophils and macrophages from oxidative stress [19,20], (ii) to reduce atherogenicity in streptozotocin diabetic rats [21] and (iii) to reduce the streptozotocin-induced oxidative damage to cardiac and renal tissues in rats [22].

On the whole, these data prompted us to analyze the efficacy of this molecule, as well as of the crude mango extract Vimang® against oxidative damage to erythrocytes. Along this line, this study was designed to determine the effect of Vimang and mangiferin, the main polyphenols present in mango extracts, on the MDA release in erythrocytes as well as on the energy metabolism after hydrogen peroxide exposure ex vitro on red blood cells from healthy individuals. EGCG [23] was used as a reference compound in order to compare the activity of Vimang and mangiferin.

2. Materials and methods

2.1. Plant Material

M. indica was collected from a cultivated field located in the region of Pinar del Rio, Cuba. Voucher specimens of the plant (Code: 41722) were deposited at the Herbarium of Academy of Sciences, guarded by the Institute of Ecology and Systematic from Ministry of Science, Technology, and Environmental, Havana, Cuba. Stem bark extract of *M. indica* was prepared by decoction and concentrated by evaporation and spray dried to obtain a fine brown powder, which was used as the standardized active ingredient of Vimang® formulations. It melts at 210–215 °C with decomposition.

The extract contains a defined and standardized mixture of components such as polyphenols, terpenoids, steroids, fatty acids and microelements [14] and it was provided by the Center of Pharmaceutical Chemistry, Havana, Cuba. A phytochemical investigation of mango stem bark extract has led to the isolation of seven phenolic constituents (namely gallic acid, 3,4-dihydroxy benzoic acid, gallic acid methyl ester, gallic acid propyl ester, mangiferin, (+)-catechin, (−) epicatechin, benzoic acid and benzoic acid propyl ester, with mangiferin as the main polyphenol present). Mangiferin content was determined by HPLC methods [13] and it represents approximately 13% of powdered extract.

Mangiferin was purified from a 5 mg/mL solution of the lyophilized powder mentioned above. It was extracted with methanol (85%) and crystallized in aqueous ethylacetate as described in [24]. Further analysis demonstrated that mangiferin purity was about 95% by high performance liquid chromatographic (HPLC) analysis using PDA detector (254 nm) and a mobile phase of acetonitrile and 3% acetic acid (16:84) [22].

Purified EGCG was purchased from Sigma Chemical.

2.2. Induction of oxidative stress to human erythrocytes

The simplicity, availability and ease of isolation make erythrocytes an excellent model for membrane studies and redox analysis [25]. Free radical damage to erythrocytes was performed as described previously [26]. In brief, peripheral venous blood samples were obtained from healthy volunteers and collected into heparinized tubes. After 10 min centrifugation at 1853×*g* and 4 °C, carried out within 15 min of withdrawal, erythrocytes were washed twice with a large volume of 10 mM glucose supplemented PBS. After the second wash, packed erythrocytes were gently resuspended with PBS-glucose to obtain a 5% haematocrit and preincubated at 37 °C for 10 min in the presence of 1 mM NaN_3 (to inhibit catalase activity). Subsequently, they were divided into various aliquots of 1.6 mL for each experimental treatment. All of these, except the controls tubes, were challenged with different concentrations of H_2O_2 (1, 2, 5 and 10 mM) with or without the addition of different concentrations of the natural extract Vimang (0.1, 10, 50 and 100 μg/mL), its main component, mangiferin (1, 10 and 100 μg/mL) and EGCG (0.01, 0.1, 1 and 10 μM) as reference compound. After 60 min at 37 °C, cells were kept for 60 s in an ice bath and then centrifuged at 1853×*g* for 10 min at 4 °C. Supernatants were divided into two parts: one was used for determining haemoglobin concentration, the other was deproteinized using 70% $HClO_4$ (10:1, v/v) to measure the concentration of metabolites released in the incubation medium. Preliminary experiments demonstrated that in our conditions the highest non-haemolytic H_2O_2 concentration was 10 mM. Packed erythrocytes were deproteinized by adding ice-cold 1.2 M $HClO_4$ (1:2, w/w). Both deproteinized red blood cells and supernatants were centrifuged at 20,690×*g* for 10 min at 4 °C, neutralized by adding 5 M K_2CO_3 in ice, filtered through a 0.45 μM Millipore-HV filter and then analyzed

by HPLC (100 μL) for the simultaneous direct determination of malondialdehyde (MDA) and adenine nucleotides [27].

2.3. Biochemical analysis

Concentrations of MDA and high-energy phosphates were determined on 100 μL of perchloric acid extract by an ion-pairing HPLC method [27] using a Vydac 250 × 4.6 mm, 5-μm particle size column, with its own guard column (Eka Chemicals AB, Bohus, Sweden), and using tetrabutylammonium hydroxide as the pairing reagent. Briefly, separation of different metabolites was obtained by forming a step gradient (adapted to the column length increase with respect to the original method [27]) with two buffers of the following composition: buffer A, 10 mM tetrabutylammonium hydroxide, 10 mM KH_2PO_4, 0.25% methanol pH 7.00; buffer B, 2.8 mM tetrabutylammonium hydroxide, 100 mM KH_2PO_4, 30% methanol pH 5.50. The gradient was: 10 min 100% buffer A; 3 min 90% buffer A; 10 min 70% buffer A; 12 min 55% buffer A; 15 min 45% buffer A; 10 min 25% buffer A; 5 min 0% buffer A. The flow rate throughout chromatographic runs was 1.2 mL/min and the column temperature was kept at a constant 23 °C by using water-jacketed glassware. The HPLC apparatus consisted of a Surveyor LC Pump (ThermoFinnigan Italia, Rodano, Milan, Italy) connected to a Surveyor PDA Detector (ThermoFinnigan Italia) at 200–300 nm. Acquisition and analysis of data were performed using the ChromQuest program (ThermoQuest Italy). Comparison of areas, retention times and absorbance spectra of the peaks of sample chromatograms with those of freshly prepared ultrapure standards allowed the calculation of the concentration of the various compounds at 267 nm (the maximum of MDA absorbance spectrum) and the identification of the different metabolites. Haemoglobin and percentage haemolysis were calculated by standard haematological techniques [28] using a Jasco-685 double beam spectrophotometer.

Energy charge potential (ECP) was calculated according to [29] by means of the following relationship:

$$ECP = ATP + 0.5ADP/\Sigma NT$$

where ΣNT (=ATP+ADP+AMP) is the sum of adenine nucleotides.

2.4. Statistical analysis

Data were entered into the GraphPad Prism statistical analysis program (GraphPad, San Diego). Comparison of results from various incubations conditions were done by one-way analysis of variance (ANOVA). Analysis *a posteriori* was carried out using Dunnett's, Bonferroni's or Tukey's tests whenever significant effects of different molecules were found on MDA production. In particular, unequal sample size correction, if necessary, was always applied to Dunnett's test; Bonferroni's test was used for multiple comparison and Tukey's test was used to analyze media and standard deviations in different experiments.

3. Results

3.1. Analysis of metabolic decay

In order to study the effects of antioxidants on oxidative stress, we analyzed both metabolic decay and lipid peroxidation in human erythrocytes. A preliminary paper [27] demonstrated that with a simple ion-pairing HPLC analysis, it is possible to simultaneously detect the amount of adenine nucleotide derivatives and malondialdehyde so as to obtain information about energy metabolism and the peroxidative damage. A typical elution pattern of PCA extract from erythrocytes is shown in Fig. 1, where the assignment of peaks to the corresponding compounds has been performed by comparing the retention times of the samples with respect to those obtained with a standard mixture. On the basis of the results obtained, we compared the effects of different concentrations of mangiferin, Vimang extract and EGCG on energy charge of red blood cells.

Fig. 2 shows that the main phosphorylated compounds necessary for all the energy requiring reactions of erythrocyte

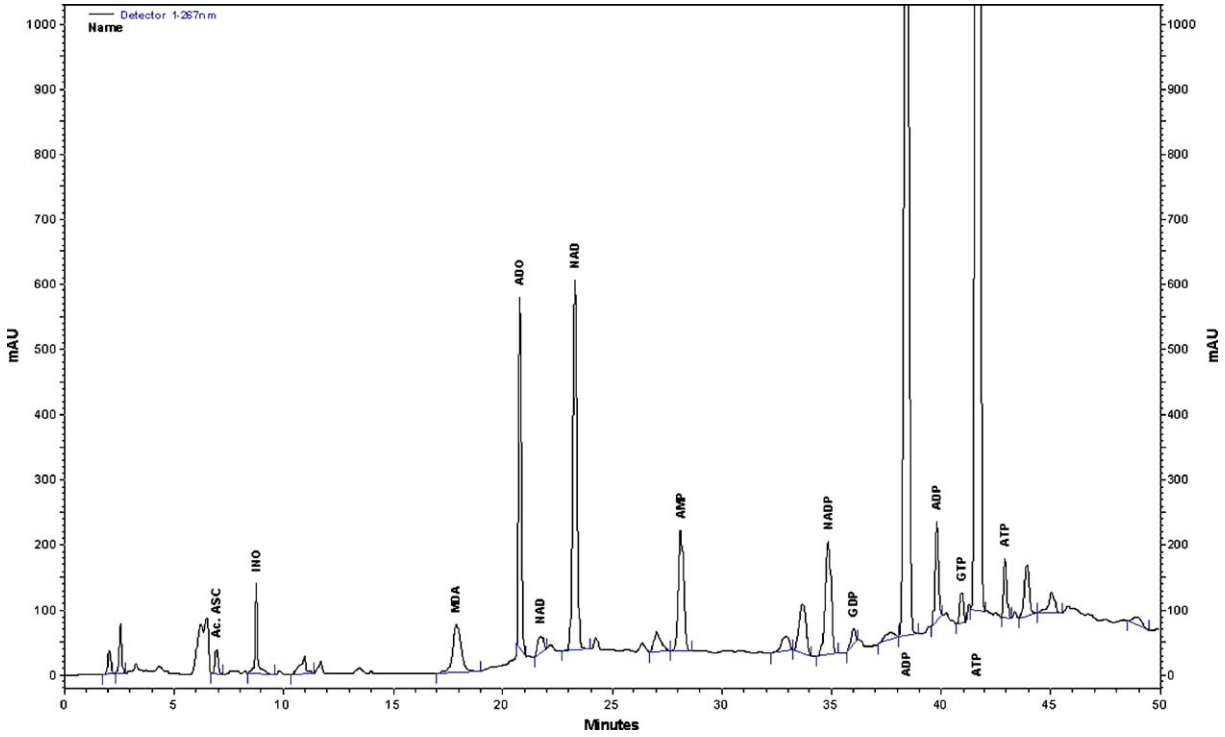

Fig. 1. A typical chromatogram obtained by using ion-pairing HPLC column as described in Materials and methods. Results are referred to 20 μL of PCA extract of control RBC.

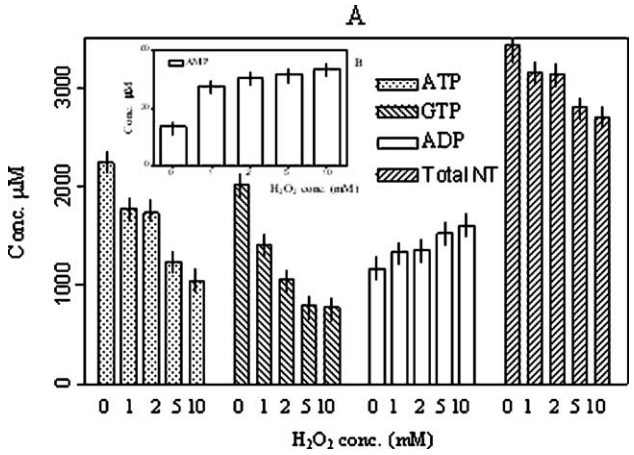

Fig. 2. Effects of H_2O_2 on ATP, GTP, ADP, total nucleotides (Total NT) and AMP concentrations. Data reported are referred to results obtained in a typical experiment performed in triplicate. Standard error never exceeded 5% of the value of each column. Different concentrations of H_2O_2 are reported in the abscissa. Differences in ATP, GTP ADP and total NT concentrations were significant ($P<0,05$) between 0 and 10 mM H_2O_2 concentrations.

metabolism, i.e., ATP and GTP, were significantly reduced after H_2O_2 challenge in a dose-dependent manner, whereas usually a dose-dependent decrease of total nucleotidic pool was observed upon H_2O_2 addition (3438 µM in controls vs. 2700 µM in 10 mM H_2O_2 treated RBC). The oxidative stress activates one major route of pyridine nucleotide catabolism (i.e., protein ADP-ribosylation) without acute inhibitory effect on the other one (i.e., cleavage by NAD glycohydrolase) [30]. The increase of NAD observed in our conditions, ranging from 580 µM in untreated cells to 934 µM in 10 mM H_2O_2-treated cells (data not shown) further supports the occurrence of an overproduction of oxygen radicals observed upon H_2O_2 addition.

The energy potential charge, which in erythrocytes and other cells is roughly close to unit (0,823 in our experiments) decreases in a dose-dependent manner when H_2O_2 is added, falling to 0.684 when H_2O_2 concentration is 10 mM (Fig. 3).

3.2. Protective effects of Vimang, mangiferin and EGCG on energy charge potential

The treatment of erythrocytes with increasing concentrations of Vimang (0.10–100 µg/mL), mangiferin (1–100 µg/mL) and EGCG (0.01–10 µM) was significantly effective in inhibiting the depletion of high energy phosphates. In particular, over a concentrations range between 0.1 and 100 µg/mL, Vimang restores, in a dose-dependent way, the ATP, GTP an total NT content of RBC, thus confirming its anti oxidant activity (Fig. 4, panel A). This protective activity on total NT concentration also mirrors that on NAD and ADP production, induced by ROS (Fig. 4, panel B). Energy potential charge was also preserved in a dose-dependent way by Vimang addition, further confirming the antioxidant activity of the mixture (Fig. 4, panel C).

Similar results have been obtained by using mangiferin. Results shown in Fig. 5 (panel A) demonstrate the antioxidant activity of this compound measured both as ATP, GTP and total NT savings associated to a decrease of NAD and ADP content (Fig. 5, panel B), which had been increased after H_2O_2 addition. Energy charge potential, decreased in H_2O_2-treated RBC, was also restored after addition of mangiferin in a dose-dependent way.

EGCG, a well-known antioxidant compound, was evaluated for its protective activity on nucleotidic pool and energy charge potential. Results demonstrate that this molecule is highly active and is able to significantly reduce, even at low concentrations, the oxidative stress induced by H_2O_2 on nucleotides (Fig. 6, panel A), on ADP and NAD production (Fig. 6, panel B) and on energy charge potential (Fig. 6, panel C).

On the whole, both total NT concentration and ECP, modified after H_2O_2 addition, were restored in a dose-dependent way by all compounds used, thus confirming the protective effects of the molecules used in our study.

3.3. Analysis of MDA production

Preliminary experiments demonstrated that MDA production followed a time-dependent increase after H_2O_2 addition in a dose-dependent way (data not shown).

The effect of Vimang and its main component, mangiferin, on lipid peroxidation in RBC is shown in Fig. 7. Total concentration of MDA (i.e., intracellular+extracellular) increased after addition of 10 mM H_2O_2 from non detectable levels in the control samples to approximately 250 µM. Vimang was very effective in reducing erythrocytes membrane lipoperoxidation; thus, even at a concentration as low as 0.1 µg/mL it was capable of bringing about a more than 2-fold decrease of MDA concentration. Similar results have been obtained by using mangiferin and EGCG.

3.4. Activity of Vimang, mangiferin and EGCG on haemolysis in presence of H_2O_2

MDA can decrease red blood cells fluidity. The lowered membrane fluidity compromises erythrocyte deformability

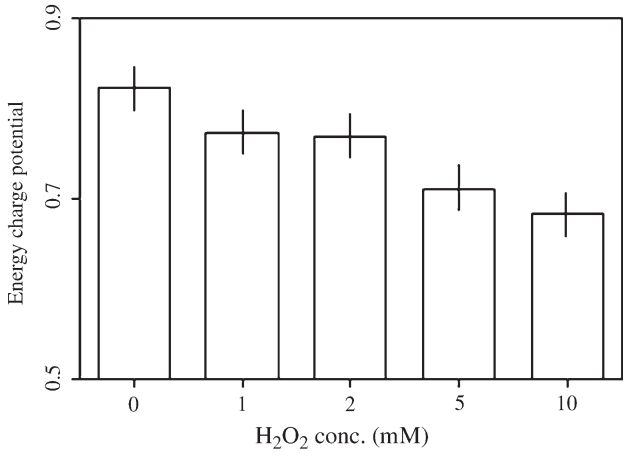

Fig. 3. Effects of different concentrations of H_2O_2 on energy charge potential. Energy charge is referred as Energy charge=ATP+1/2 ADP/ATP+ADP+AMP. Differences in energy charge was significant ($P<0,05$) between 0 and 10 mM H_2O_2 concentrations.

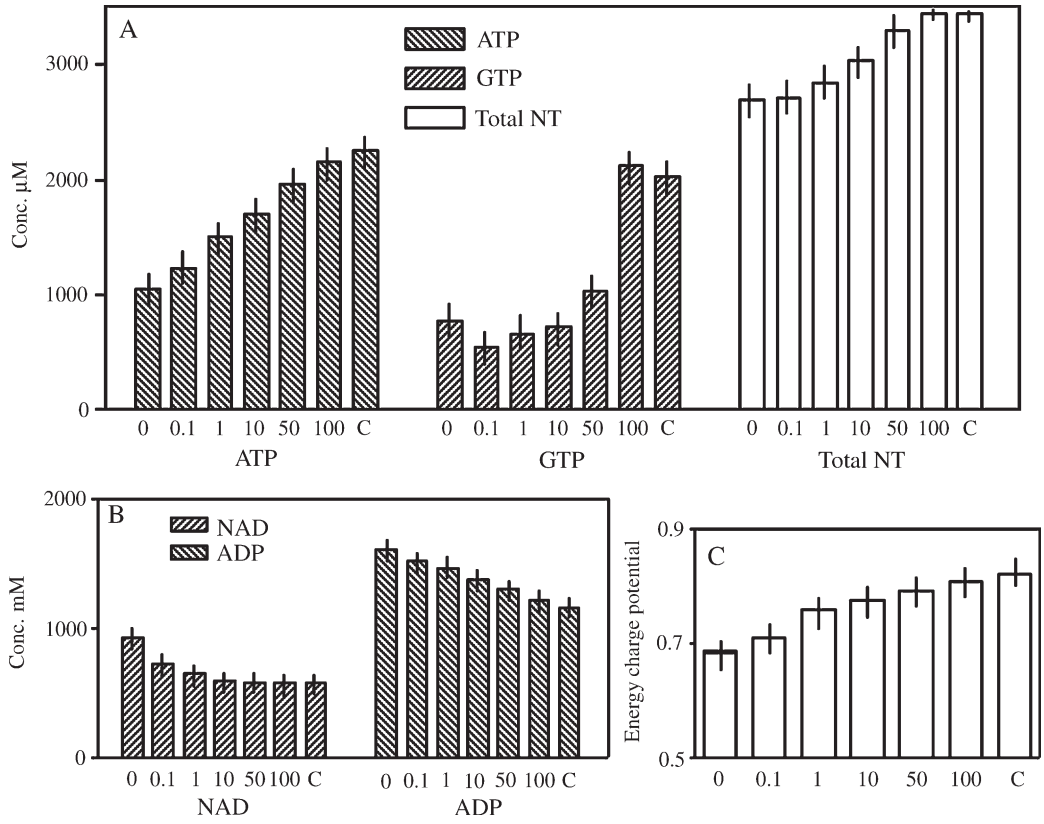

Fig. 4. Effect of Vimang at different concentrations on energy metabolism. Different concentrations (µg/mL) of Vimang, as reported in the abscissa, were added to 10 mM H_2O_2 treated RBC, as described in Materials and methods. C column is referred to untreated RBC. Panel A: ATP, GTP and total NT concentrations; panel B: NAD and ADP concentrations; panel C: energy charge potential. Each column represents the mean±S.E. of three different blood samples expressed as µM. Differences in ATP, GTP, total NT, NAD, ADP concentrations and energy charge were significant ($P<0.05$) between 0 and 100 µg/mL Vimang concentrations. No statistically significant difference between 100 µg/mL Vimang and control RBC has been observed.

which in turn disturbs oxygen delivery to the tissues and enhances haemolysis which could be a marker of oxidative stress. In our conditions we observed a dose-dependent increase on haemoglobin release in H_2O_2–treated erythrocytes. The amount of haemolysis was reduced in a dose-dependent manner in the presence of the different test substances used in our study, thus further confirming their activity on lipid peroxidation and membrane fluidity (Fig. 8). In particular, a reduction of haemolysis by about 50% was observed when Vimang was used at a concentration of 100 µg/mL. Even higher haemolysis reduction has been observed when EGCG was used.

3.5. Effects of Vimang, mangiferin and EGCG on ascorbate production

Ascorbate is a natural antioxidant able to reduce the activity of free radicals. Since ascorbate is involved in vitamin E redox cycling and vitamin E is the main membrane-bound lipid peroxidation chain breaker, a decrease in ascorbate concentration might be responsible for an enhanced MDA production. Ascorbate amount rapidly decreases in the presence of H_2O_2, thus favouring lipid peroxidation. The concentration of ascorbate as a sensitive intracellular antioxidant [31] was checked and it turns out that Vimang, mangiferin and EGCG markedly enhance, in a dose-dependent way, the production of RBC–mediated ascorbate, which had been decreased upon H_2O_2 addition; these data further confirm the protective effects that these molecules can exert on erythrocytes (Fig. 9). In particular, addition of 100 µg/mL Vimang or mangiferin and 10 mM EGCG fully restores ascorbate production after H_2O_2-induced oxidative stress.

4. Discussion

The aim of the present work was to prove the phytotherapeutical significance of an officinal and popular medicinal extract and its main ingredient, on the basis of their antioxidant activity due to their influence on pathological free radical reactions. Experimental methods were planned and developed in order to measure the antioxidant, free radical scavenging and membrane protecting activities and to monitor the capacity of these natural compounds to reduce the extend of lipid peroxidation. Some previous “in vivo” experiments demonstrated that Vimang is a better scavenger of ROS and a more efficient inhibitor of oxidation tissue damage than vitamin C, vitamin E, β-carotene and mangiferin [12]. Mangiferin, constituting approximately 10% of the whole Vimang extract [13], was also identified as a ROS scavenger.

Different models have been proposed to detect both the effects of reactive oxidizing species and the activity of natural

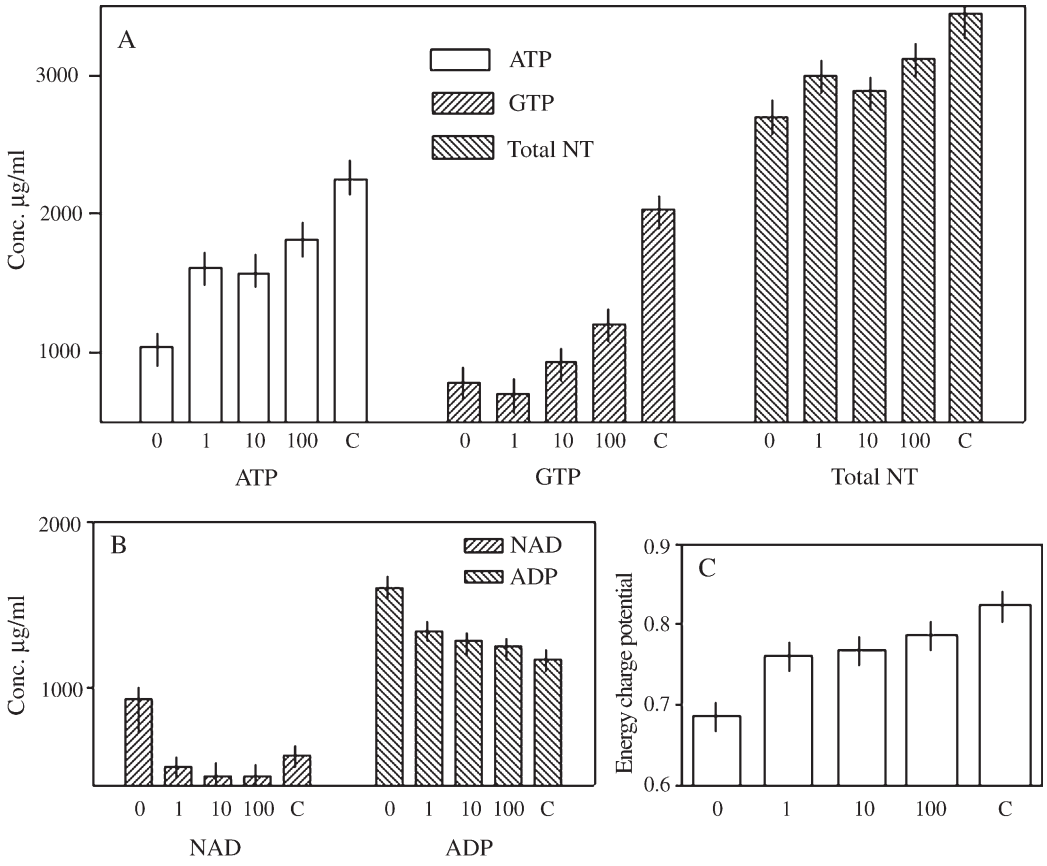

Fig. 5. Effect of mangiferin at different concentrations on energy metabolism. Different concentrations (μg/mL) of mangiferin, as reported in the abscissa, were added to 10 mM H_2O_2-treated RBC, as described in Materials and methods. C column is referred to untreated RBC. Panel A: ATP, GTP and total NT concentrations; panel B: NAD and ADP concentrations; panel C: energy charge potential. Each column represents the mean±S.E. of three different blood samples expressed as μM. Differences in ATP, GTP, total NT, NAD, ADP concentrations and energy charge were significant ($P<0.05$) between 0 and 100 μg/mL mangiferin concentrations. No statistically significant difference between 100 μg/mL mangiferin and control RBC has been observed.

and synthetic scavengers; among them, erythrocytes rapidly became a useful general model to evaluate the effects of ROS and antioxidants on a very accurate cellular system. Besides the practical implications of this cell system, there is a physiological interest because erythrocytes represent an important component of the antioxidant capacity of blood, comprising the glutathione system and intracellular enzymes, such as superoxide dismutase and catalase. ATP and energy substrate concentrations in the cell are not random; they are part of stress response of the cell and an abrupt rise in energy demand, due to the increased ROS concentration, induces the adenylate pool to respond first by a decrease in its size. As a consequence of the imbalance between phosphorylating and dephosphorylating processes in the presence of oxidants, such as H_2O_2, both total NT and ECP were also remarkably reduced in a dose-dependent manner. In our conditions, after 1 h incubation at 37 °C in the presence of the highest H_2O_2 concentration (10 mM), about 46% ATP depletion was observed with a concomitant significant ADP increase (37%). AMP concentration rose from 20.45 μM to 50.1 μM and GTP concentration decreased from 2.025 mM in control to 781 μM when 10 mM H_2O_2 was used to trigger oxidative stress. Adenosine, a marker of ATP degradation, also increased during H_2O_2 incubation, in a dose-dependent manner, from 320 μM for control erythrocytes to 1.36 mM when H_2O_2 was used at a concentration of 10 mM.

On the whole, these observations could be explained by the tetravalent reduction of the reactive oxygen species (produced by H_2O_2) which, in turn, consumes ATP, thus progressively reducing the energy charge. The rapid decrease in ATP content is further associated to a loss of ability for a correct reduction of oxygen catalysis. This phenomenon, in combination with the oxidation of hypoxanthine to xanthine and uric acid catalyzed by xanthine oxidase, has been proposed as one of the main intracellular sources of superoxide anions. Although not particularly toxic, this molecule may be transformed, through different mechanisms, into various highly reactive and toxic oxygen radicals, such as the strong oxidant hydroxyl radical $OH^{\cdot}$. Therefore, antioxidants which are able to impair the formation of oxygen reactive species could block ATP consumption, thus maintaining in a dose-dependent way the energy charge ≈1.0.

At all concentrations used, Vimang, its main component mangiferin and EGCG have been shown to be able to scavenge ROS, thus inhibiting all those processes leading to RBC damage, energy charge decrease and membrane destabilization. In particular, all molecules used restore, in a dose-dependent way, the ATP, GTP and total NT content of RBC, thus

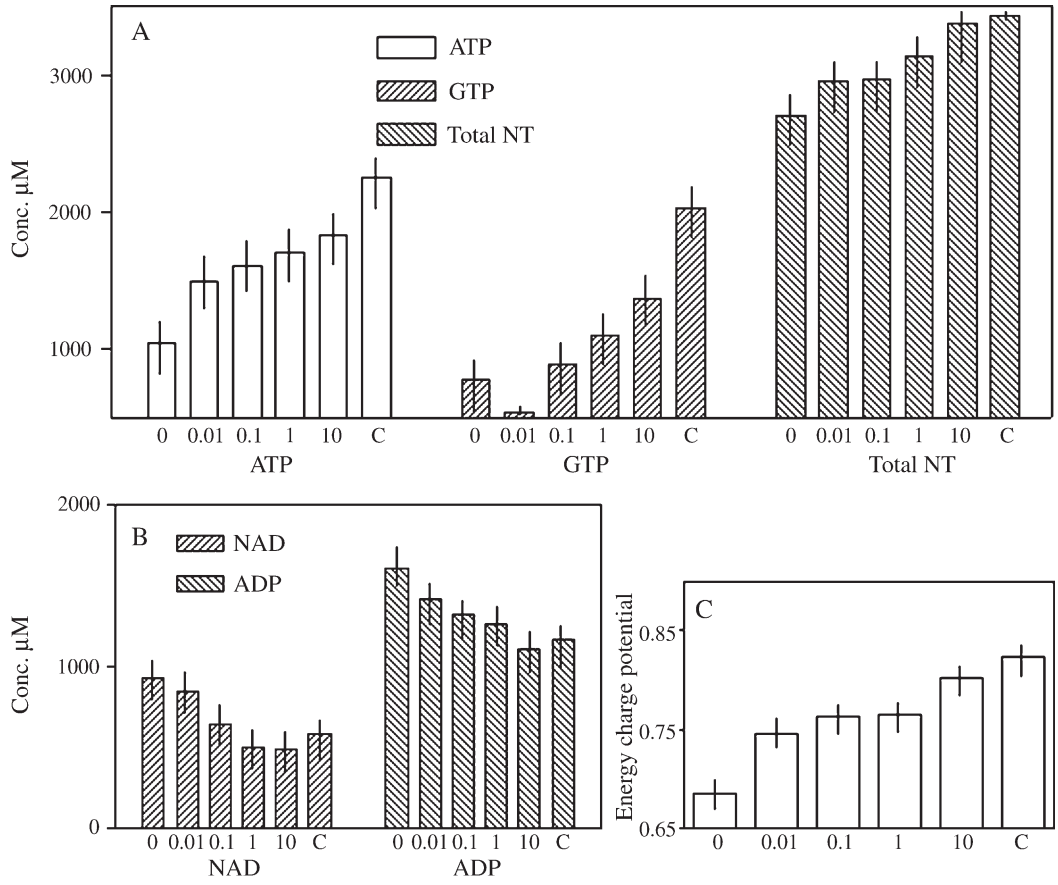

Fig. 6. Effect of EGCG at different concentrations on energy metabolism. Different concentrations (µg/mL) of EGCG, as reported in the abscissa, were added to 10 mM H_2O_2 treated RBC, as described in Materials and methods. C column is referred to untreated RBC. Panel A: ATP, GTP and total NT concentrations; panel B: NAD and ADP concentrations; panel C: energy charge potential. Each column represents the mean±S.E. of three different blood samples expressed as µM. Differences in ATP, GTP, total NT, NAD, ADP concentrations and energy charge were significant ($P<0.05$) between 0 and 10 µg/mL EGCG concentrations. No statistically significant difference between 10 µg/mL EGCG and control RBC has been observed.

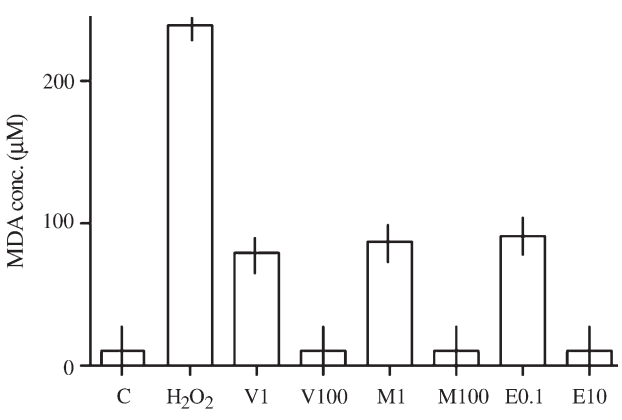

Fig. 7. Effect of different compounds on MDA production. C: untreated RBC; H_2O_2: 10 mM H_2O_2-treated RBC; V1: H_2O_2-treated RBC incubated with 1 µg/mL Vimang; V100: H_2O_2-treated RBC incubated with 100 µg/mL Vimang; M1: H_2O_2-treated RBC incubated with 1 µg/mL mangiferin; M100: H_2O_2-treated RBC incubated with 100 µg/mL mangiferin; E0.1: H_2O_2-treated RBC incubated with 0.1 µM EGCG; E10: H_2O_2-treated RBC incubated with 10 µM EGCG. Each column represents the mean±S.E. of three different blood samples expressed as µM. Differences between V1 vs. V100, M1 vs. M100 and E0,1 vs. E10 were significant ($P<0.05$).

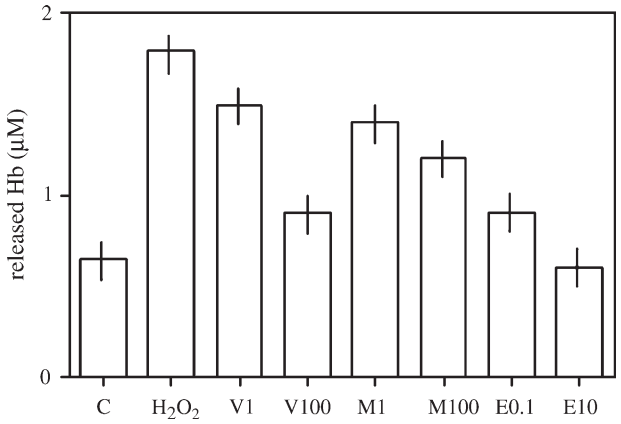

Fig. 8. Effect of different compounds on H_2O_2-induced RBC haemolysis. C: untreated RBC; H_2O_2: 10 mM H_2O_2-treated RBC; V1: H_2O_2-treated RBC incubated with 1 µg/mL Vimang; V100: H_2O_2-treated RBC incubated with 100 µg/mL Vimang; M1: H_2O_2-treated RBC incubated with 1 µg/mL mangiferin; M100: H_2O_2-treated RBC incubated with 100 µg/mL mangiferin; E0.1: H_2O_2-treated RBC incubated with 0.1 µM EGCG; E10: H_2O_2-treated RBC incubated with 10 µM EGCG. Each column represents the mean±S.E. of three different blood samples expressed as µM. Differences between V1 vs. V100 and E0,1 vs. E10 were significant ($P<0.05$).

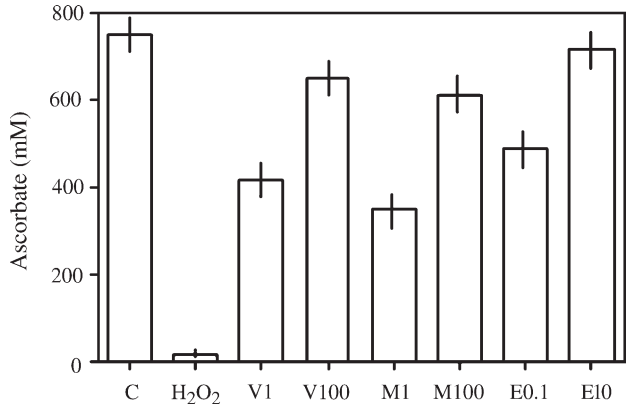

Fig. 9. Effect of different compounds on ascorbate production in H_2O_2-treated RBC. C: untreated RBC; H_2O_2: 10 mM H_2O_2-treated RBC; V1: H_2O_2-treated RBC incubated with 1 μg/mL Vimang; V100: H_2O_2 treated RBC incubated with 100 μg/mL Vimang; M1: H_2O_2-treated RBC incubated with 1 μg/mL mangiferin; M100: H_2O_2-treated RBC incubated with 100 μg/mL mangiferin; E0.1: H_2O_2-treated RBC incubated with 0.1 μM EGCG; E10: H_2O_2-treated RBC incubated with 10 μM EGCG. Each column represents the mean ± S.E. of three different blood samples expressed as μM. Differences between V1 vs. V100, M1 vs. M100 and E0,1 vs. E10 were significant ($P<0.05$). All treatment resulted in a significant increase ($P<0.05$) of ascorbate concentrations vs. H_2O_2 treated RBC.

confirming their anti-oxidant activity (Figs. 4–6). This protective activity on total NT concentration mirrors also the NAD and ADP production, induced by ROS. Energy potential charge was also preserved in a dose-dependent way, further confirming the antioxidant activity of the mixture (Fig. 4, panel C). Scavenging activity of the molecules used in the study was further confirmed by membrane stabilization, clearly indicating that ROS can damage RBC membrane by acting on membrane phospholipids. Addition of Vimang, mangiferin and EGCG significantly reduces this damage, thus restoring membrane fluidity and stability. It must be pointed out that green tea polyphenol epigallocatechin gallate (EGCG) has been proposed as a cancer chemopreventive and several studies have shown that EGCG can act as an antioxidant by trapping peroxyl radicals and inhibiting lipid peroxidation. This compound blocks lipid peroxidation in erythrocyte membranes induced by t-butylhydroperoxide and it protected ATPases against t-BHP-induced damage [23]. This paper describes, for the first time, the protective activity of this molecule on nucleotides pool and energy charge, thus confirming its strong antioxidant and scavenger activity on a very sensitive system such as RBC challenged with H_2O_2.

Erythrocytes and erythrocyte membrane have a high ratio of polyunsaturated fatty acids to total lipids, indicating susceptibility to lipid peroxidation; moreover, RBC are highly vulnerable to lipid peroxidation due to constant exposure to high oxygen tension and the presence of high Fe ion concentrations [32]. Extensive investigations demonstrated the key role of oxidative stress and iron release in a reactive form causing membrane protein damage via the Fenton reaction and hydroxyl radical production. In the absence of an efficient protection by antioxidant factors (such as flavonoids), oxidative stress is responsible for iron release in reactive form, bringing about red cells haemolysis [33]. A study performed using a spectrophotometric titration technique [34] revealed that tea polyphenols (TPP) were capable of binding ferric ion in a stoichiometric amount to form a redox inactive Fe–TPP complex. Quantitative analysis suggests that one or more major catechins from the TPP preparations are the likely iron binding compounds accounting for the antioxidant effects of TPP on red blood cells. Recent investigations have demonstrated that Vimang is able to prevent iron mediated mitochondrial damage by means of oxidation of reduced transition metals required for the production of superoxide and hydroxyl radicals and direct free radical scavenging activity. This study describes the iron-complexing ability of Vimang as the primary mechanism for protection of rat liver mitochondria against Fe^{2+}–citrate-induced lipoperoxidation; on the other hand, mangiferin could form a complex with Fe^{2+}, accelerating the Fe^{2+}oxidation and the formation of more stable $Fe3^{+}$–polyphenol complexes, which are unable to participate in Fenton-type reactions, and the lipoperoxidation propagation phase [35].

Dietary antioxidants are believed to protect humans from disease and aging, since they play a major role in maintaining the homeostasis of the oxidative balance. Indeed, a large number of previous studies has also reported enhanced protection by polyphenols on RBC [36]. Plant-derived phenols are reported to have a broad spectrum of free-radical scavenging, antioxidant and protective activities [37–41]. In other studies red wine polyphenols, containing 3.5 mM gallic acid equivalent (GAE) of phenolic compounds, prevented oxidative modifications on the RBC system, such as haemolysis and lipid peroxidation. The protective effect was less apparent when red blood cells were incubated with wines containing lower levels of polyphenols [42]. (−)Epicatechin has been shown to enhance antioxidative defense systems in diabetic erythrocytes [43].

We should underline that mangiferin constitutes approximately 10% of the whole extract and, considering this, it might be possible that the efficacy of Vimang at 100 μg/mL could be similar to that obtained for the pure mangiferin at 10 μg/mL. EGCG, in our experimental model, was found to be more active than mangiferin, thus indicating the importance and the possible synergistic effects due to other polyphenols in the extract.

By-products of lipid peroxidation are low-molecular weight compounds, such as carbohydrates (ethane and pentane) and aldehydes, e.g., MDA, which is the by-product of both peroxidation of phospholipids and the activation of the arachidonate cycle. Therefore, it is generally regarded as a marker of peroxidative damage induced in cell membranes by both physical and chemical oxidative stresses, rendering its determination in biological samples particularly interesting [44]. Although the inhibitory activity of Vimang and mangiferin on MDA production has been described previously [11,12], its determination in the present work by HPLC technique furnishes more reliability and reproducibility to the obtained results. Nevertheless, these products where studied for the first time in a new context, erythrocytes system, giving another explanation of

the beneficial effects of Vimang consumption by patients suffering of oxidative stress.

Vitamin C is considered to be one of the most powerful, least toxic natural antioxidants. It is found in high concentrations in many tissues; human plasma contains about 60 mM ascorbate [31]. After interaction with ROS, ascorbate is oxidized to dehydro-ascorbate via the intermediate ascorbyl free radical. Dehydro-ascorbate is recycled back to ascorbic acid by the enzyme dehydro-ascorbate reductase. Thus, dehydro-ascorbate is found at only very low levels as compared to ascorbate. As a scavenger of ROS, ascorbate has been shown to be effective against H_2O_2, the hydroxyl radical, the superoxide radical anion, singlet oxygen and in aqueous solutions it also scavenges reactive nitrogen oxide species efficiently, preventing the nitration of target molecules. We also determined the ascorbic acid concentration in the analyzed samples as an attempt to elucidate the mechanisms of the antioxidant action of the extract. The enhancement of this molecule in peroxidized cells treated with the extract might indicate the activation of antioxidant defenses by all molecules used in the study. Since Vimang has been studied in different in vitro and in vivo systems, where it elicited a clear antioxidant effect, its action in human beings might be predictable. A sign of this has been a clinical trial where Vimang supplementation on HIV patients shows evidence of improved antioxidant status and reduced oxidative damage [45].

The results presented herein, allow us to conclude that besides of the aforementioned antioxidant effects of Vimang, mangiferin and EGCG, they exert an important role in the protection of erythrocytes from ROS production, contributing to the integrity and functionality of these cells. Their protective activities against erythrocyte damage constitutes another reason to consider them as a promising therapeutic agents for a great number of pathologies where cell oxidative stress is implicated.

Acknowledgment

A la memoria de Janet Rodríguez, investigadora que murió inesperadamente el 3 de diciembre del 2005 con solo 28 años, cuando todavía este trabajo no había sido publicado. Janet simboliza el talento, la sencillez y la belleza de una joven cubana excepcional, querida por todos sus colegas y amigos, permanecerá por siempre en el corazón y la memoria de quienes tuvimos el inmenso placer de conocerla y compartir con ella su virtuosismo y apasionada dedicación al trabajo científico.

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Modulation of the proteolytic activity of matrix metalloproteinase-2 (gelatinase A) on fibrinogen

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The proteolytic processing of bovine fibrinogen by MMP-2 (gelatinase A), which brings about the formation of a product unable to form fibrin clots, has been studied at 37 °C. Catalytic parameters, although showing a somewhat lower catalytic efficiency with respect to thrombin and plasmin, indeed display values indicating a pathophysiological significance of this process. A parallel molecular modelling study predicts preferential binding of MMP-2 to the β-chain of fibrinogen through its haemopexin-like domain, which has been directly demonstrated by the inhibitory effect in the presence of the exogenous haemopexin-like domain. However, the removal of this domain does not impair the interaction between MMP-2 and fibrinogen, but it dramatically alters the proteolytic mechanism, producing different fragmentation intermediates. The investigation at various pH values between 6.0 and 9.3 indicates a proton-linked behaviour, which is relevant for interpreting the influence on the process by environmental conditions occurring at the site of an injury. Furthermore, the action of MMP-2 on peroxynitrite-treated fibrinogen has been investigated, a situation possibly occurring under oxidative stress. The chemical alteration of fibrinogen, which has been shown to abolish its clotting activity, brings about only limited modifications of the catalytic parameters without altering the main enzymatic mechanism.

Key words: fibrinogen, fragmentation, gelatinase A, kinetics, molecular modelling, pH-dependence.

INTRODUCTION

MMPs (matrix metalloproteinases) are a class of Ca^{2+}- and Zn^{2+}-dependent endopeptidases, displaying an active site characterized by a Zn^{2+} atom co-ordinated to three histidine residues [1]. They show a multi-domain structural organization, usually made up of a propeptide domain (which is removed upon enzyme activation), a catalytic domain and a haemopexin-like domain, which are connected by a hinge domain. In addition, a MMP subclass composed of two MMPs, MMP-2 and MMP-9 (gelatinases A and B respectively), displays a unique collagen-binding domain, called a fibronectin-like domain, inserted on the catalytic domain and consisting of three 58-amino-acid fibronectin type II-like modules [2].

These enzymes, which are secreted as inactive zymogens, have the capacity of degrading several proteins of the extracellular matrix, participating in most of the tissue remodelling phenomena [3]. Furthermore, since they are released in the plasma from the polymorphonuclear leukocytes [4], it is very probable that they come into close contact with plasma proteins, which can then become physiological substrates for MMPs. As a matter of fact, a growing interest has been addressed toward the role of MMPs in the neovascularization events which play a key role in several physiological and pathological processes, from tumour dissemination to wound healing. In particular, MMP-2 is involved in a process termed the 'angiogenic switch' [5,6], which consists of the induction of a new vasculature by degrading the vascular basement membrane and the extracellular matrix, in order to allow endothelial cells to migrate into the perivascular space. This process can occur at different stages of tumour progression, depending on tumour type and environment. Premalignant lesions may also induce neovascularization and it has been shown that, during the neovascularization event, the role of several MMPs appears to be that of pericellular fibrinolysins [7], some of them being involved in the degradation of fibrinogen and cross-linked fibrin necessary for the migration of endothelial cells [8,9]. Therefore the enzymatic action by MMPs on components of the blood coagulation cascade is relevant both from the physiological and the pathological standpoint.

Fibrinogen is a 340 kDa dimeric glycoprotein, present in the blood, which is composed of six polypeptide chains $(\alpha\beta\gamma)_2$, joined by 29 disulfide bridges within the N-terminal E-domain, forming an elongated 45 nm structure consisting of two outer D-domains connected through a central E-domain by a coiled–coil segment. During blood coagulation, thrombin converts fibrinogen into fibrin monomers, which associate into staggered overlapping two-stranded fibrils and these domains contain binding sites which participate in the fibrinogen conversion into fibrin [10]. However, previous data clearly indicate a correlation between the activity of MMP-2 and MMP-9 and that of fibrinogen in the neovascularization process, since it has been shown that inhibition of the gelatinase action brings about an accumulation of unprocessed fibrinogen and fibrin, and this is accompanied by a blocking of the neovascularization process [11]. Further, during oxidative stresses connected to the blood vessels damage and their repair, a dramatic enhancement of the production of reactive oxygen species and reactive nitrogen species is observed, leading to vascular injury responsible for the development of atherosclerosis [12]. As a matter of fact, levels of nitrated proteins have been shown to be increased in atherosclerotic lesions [13–16], being

Abbreviations used: DPA, N-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; MCA, (7-methoxycoumarin-4-yl)acetyl; MMP, matrix metalloproteinase; MMP-2, gelatinase A; MMP-9, gelatinase B.

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predictors of a risk for coronary artery disease [17]. This observation provides additional support for the previous observation that post-translational modifications of fibrinogen by reactive nitrogen species, such as peroxynitrite [18], bring about dramatic alterations in the role of fibrinogen [19].

Therefore the elucidation of the mechanism by which the enzymatic activity of MMP-2 on native and oxidized fibrinogen is modulated can be of the utmost importance for a better comprehension of the different interplay between MMPs and coagulation properties, and of their physiopathological relevance for angiogenesis. In the present study we have analysed the catalytic efficiency of whole MMP-2 on native and oxidized fibrinogen, comparing it with that of the catalytic domain alone, in order to clarify the role of different domains of MMP-2 during fibrinogen degradation. It is interesting to observe that the efficiency of the enzymatic action of MMP-2 on fibrinogen turns out to be somewhat lower than that of thrombin [20] and similar to that displayed by plasmin [21]. Moreover, we accompany this investigation with a molecular modelling of the interaction between MMP-2 and fibrinogen in order to formulate a plausible mechanism based on structural and functional information. In addition, since pH may vary significantly from neutrality in injured areas and during inflammatory responses, we have performed an analysis of the pH-dependence of catalytic parameters for the processing of the α- and β-chains of fibrinogen by MMP-2 in order to provide more information on the modulation of this relevant process by environmental conditions.

EXPERIMENTAL PROCEDURES

Materials

Bovine fibrinogen (Sigma) was dissolved in water at room temperature (25 °C) to a final concentration of 1 mg/ml. The suspension was centrifuged for 1 h at 10000 ***g*** and the supernatant containing the dissolved fibrinogen was used. The amount of substrate was quantified using the method described by Bradford [22].

Peroxynitrite was synthesized according to the protocol reported by Uppu et al. [23] and stored at − 80 °C. Bovine peroxynitrite-treated fibrinogen was prepared by treating native fibrinogen (5 mg/ml) with 40 mM of peroxynitrous acid, in 50 mM PBS (pH 7.2) and 0.1 mM DTPA (diethylenetriaminepenta-acetic acid).

MMP-2 proenzyme was either of commercial origin (R&D Systems) or a gift from Dr Chris Overall (Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, Canada), and no significant functional difference was detected between the two preparations after activation. The isolated purified MMP-2 was activated by incubating 0.1 ml of a 0.1 μg/ml progelatinase solution with APMA (*p*-aminophenyl mercuric acid; Sigma) at 37 °C for 30 min and immediately used for experiments. A control experiment using gelatin zymography showed that over the first 24 h no autocatalytic process was taking place under our experimental conditions (results not shown), as has already been described by Nagase et al. [24].

The haemopexin-like domain of MMP-2 was a gift from Dr Chris Overall (Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, Canada) and was prepared as previously reported [25].

Human recombinant MMP-2 catalytic domain (Biomol International) was dissolved in a solution of 50 mM Tris/HCl (pH 7.2), 0.1 M NaCl and 10 mM CaCl.

The quenched fluorogenic substrate MCA-Pro-Leu-Gly-Leu-DPA-Ala-Arg-NH_2 [where MCA is (7-methoxycoumarin-4-yl)acetyl and DPA is N-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl] was purchased from Calbiochem.

Plasmin enzyme and the fluorogenic peptide MUGB (4-methylumbelliferylguanidinobenzoate), an active site titrant of trypsin-like proteases, were of commercial origin (Sigma).

Activity assay

The active amount of MMP-2 was determined using gelatin zymography and using the fluorimetric assay, as previously reported [26], following the progressive decrease of hydrolysis of the quenched fluorogenic substrate MCA-Pro-Leu-Gly-Leu-DPA-Ala-Arg-NH_2 upon addition of BB-94 (batimastat), a peptidomimetic inhibitor (provided by British Biotech Pharmaceuticals), which stoichiometrically inhibits MMPs.

Digestion of fibrinogen by MMP-2 and plasmin, and associated kinetics

For substrate fragmentation kinetics, activated whole MMP-2 or plasmin were added to fibrinogen solutions at a final concentration of 10 pM, whereas the catalytic domain of MMP-2 was added to fibrinogen solutions at a final concentration of 15–20 pM. The kinetics of MMP-2 were performed in 50 mM Tris/HCl, 0.1 M NaCl and 10 mM $CaCl_2$ at 37 °C and at different values of pH, using different concentrations of bovine fibrinogen (spanning between 5.3 μM and 20 μM). The same range of substrate concentrations was used for peroxynitrite-treated fibrinogen, but in this case, as in the case of plasmin, kinetics were performed at only pH 7.1. In the case of the experiments in the presence of the haemopexin-like domain of MMP-2, 6 μM fibrinogen was equilibrated for 30 min in the presence of 200 μM haemopexin-like domain (a concentration required for the almost complete saturation of the binding site, see below), then whole MMP-2 (to a final concentration of 10 pM) was added to start the kinetic reaction.

Kinetics were performed keeping the mixtures at 37 °C and harvesting small aliquots at different time intervals. Reactions were stopped by the addition of SDS/PAGE loading buffer containing 20 mM EDTA and aliquots were frozen at − 80 °C until use. The aliquots in the reducing sample buffer were separated on SDS/PAGE (10 % gels), which were stained using 0.5 % Coomassie Blue and destained in 10 % acetic acid and 40 % methanol until substrate bands were clearly visible. The broad spectrum protein markers (BioRad) were used as molecular mass standards.

Kinetic analysis

Electrophoretic spots corresponding to different aliquots at different time intervals were analysed by a laser densitometer (LKB 2202 UltraScan) and their intensity was calibrated (in order to obtain concentration values) using standard substrate solutions. It must be pointed out that different preparations of fibrinogen displayed different ratios of the relative amount of the three chains, as determined from the intensity of electrophoretic spots on SDS/PAGE under reducing conditions. For α- and β-chains the substrate disappearance rates were derived at each fibrinogen concentration employed.

The measurement of the initial velocity referred to a time period over which less than 10 % of the substrate was degraded during the assay; this period never exceeded 10 h, which guaranteed the integrity of activated MMP-2, as reported previously by Nagase et al. [24]. Therefore even though we cannot rule out the possibility that at very long time intervals (i.e. $>$ 48 h) MMP-2 undergoes a

Table 1 Catalytic parameters for the enzymatic processing at 37 °C and pH 7.1 of native fibrinogen by the whole MMP-2, the catalytic domain of MMP-2 and plasmin and of peroxynitrite-treated fibrinogen by whole MMP-2

	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	k_{cat} (s^{-1})	K_m (M)
α-Chain			
Native fibrinogen			
Whole MMP-2	$2.29(\pm 0.23) \times 10^5$	$36.4(\pm 4.2)$	$1.59(\pm 0.18) \times 10^{-4}$
Catalytic domain MMP-2	$1.09(\pm 0.12) \times 10^4$	$0.19(\pm 0.02)$	$1.72(\pm 0.19) \times 10^{-5}$
Plasmin	$2.04(\pm 0.21) \times 10^5$	$7.7(\pm 0.9)$	$3.77(\pm 0.41) \times 10^{-5}$
Oxidized fibrinogen			
Whole MMP-2	$1.05(\pm 0.11) \times 10^5$	$3.1(\pm 0.4)$	$2.93(\pm 0.35) \times 10^{-5}$
β-Chain			
Native fibrinogen			
Whole MMP-2	$6.61(\pm 0.83) \times 10^4$	$12.5(\pm 1.4)$	$1.89(\pm 0.20) \times 10^{-4}$
Catalytic domain MMP-2	$3.59(\pm 0.42) \times 10^3$	$0.12(\pm 0.02)$	$3.27(\pm 0.41) \times 10^{-5}$
Plasmin	$2.47(\pm 0.34) \times 10^5$	$27.8(\pm 2.9)$	$1.12(\pm 0.15) \times 10^{-4}$
Oxidized fibrinogen			
Whole MMP-2	$1.34(\pm 0.15) \times 10^5$	$1.06(\pm 0.12)$	$7.93(\pm 0.88) \times 10^{-6}$
γ-Chain			
Native fibrinogen			
Plasmin	$4.96(\pm 0.6) \times 10^4$	$2.35(\pm 0.36)$	$4.74(\pm 0.57) \times 10^{-5}$

partial autolysis, an investigation outside the purpose of the present paper, the catalytic parameters reported in Table 1 refer to the behaviour observed within the first 10 h of the reaction. In any case, the analysis was limited to the time interval over which linearity of the rate was observed and a steady-state condition for the first cleavage step was ensured, a prerequisite for the subsequent analysis step. This consisted of the verification for the applicability of the Michaelis–Menten approximation to the first cleavage step, which was based on the observation of an inverse linear correlation between velocity and substrate concentration according to the Lineweaver–Burk equation to obtain the catalytic parameters k_{cat} and K_m:

$$\frac{E_0}{v} = \frac{K_m}{k_{cat}} \cdot \frac{1}{[S]} + \frac{1}{k_{cat}} \quad (1)$$

where E_0 is the total enzyme concentration, v is the actual rate (expressed as mol/s), K_m is the Michaelis–Menten equilibrium constant (expressed as mol), k_{cat} is the rate-limiting step kinetic constant (expressed as s^{-1}) and [S] is the substrate concentration.

Clotting experiments

The thrombin-induced clotting of fibrinogen after enzymatic processing by MMP-2 was measured spectrophotometrically, following the increase in absorbance at 350 nm as a function of time, in a Cary 1 dual-beam spectrophotometer, thermostatically controlled at 25 °C. Fibrinogen was incubated for 1 min in 50 mM Tris/HCl buffer (pH 8.2) and clot formation was triggered by addition of 1.2 units of human thrombin. Clotting curves were analysed as described previously [27].

Molecular modelling

Crystal structures isolated from the full-length proMMP-2 (PDB No. 1CK7) were used to model the interaction of fibrinogen with different truncated forms of MMP-2 (i.e. the autoinhibitory procatalytic domain, the catalytic domain, the catalytic domain lacking the fibronectin-like domain, the fibronectin-like domain alone and the haemopexin-like domain). The dimeric form of fibrinogen (PDB No. 1M1J) is symmetrical, so the structure used for the docking simulation was truncated to one monomer plus the N-terminal fragments of the second monomer to recreate the interface between the two identical monomers. The docking program employed for the molecular modelling was BiGGER (Biomolecular complex Generation with Global Evaluation and Ranking) [28].

RESULTS

Degradation of fibrinogen and peroxynitrite-treated fibrinogen by MMP-2

Figure 1(A) shows the three chains forming the fibrinogen molecule, and their enzymatic processing at 37 °C and pH 7.1 as a function of incubation time with whole MMP-2. It is interesting to observe that while α- and β-chains are progressively cleaved by MMP-2, the γ-chain turns out to be essentially immune from the cleavage action of MMP-2. This behaviour is significantly different from that reported for the proteolytic action on fibrinogen of MMP-8, MMP-12, MMP-13 and MMP-14 [29], since these MMPs seem to cleave the α-chains first and only at longer time intervals does the cleavage of the β- and γ-chains take place. However, it must be pointed out that the data in Figure 1(A) refer to whole MMP-2, whereas previous observations [29] were made using the catalytic domains of MMP-8, MMP-12, MMP-13 and MMP-14. An immediate comparison between the kinetics reported in the present paper and that reported by others [29] is not possible, since others have used a 1:10–1:50 enzyme/fibrinogen molar ratio whereas we have used a $1:10^4$–$1:10^5$ enzyme/fibrinogen molar ratio (see the Experimental procedures section). Nonetheless, similar to that which has been reported for the other MMPs [29], we have observed the formation of lower molecular mass fragments of the fibrinogen chains, which grow as a function of time of the incubation (Figure 1A). It is important to emphasize that these fragments do not show any clotting activity upon incubation with thrombin (see Figure 1B), suggesting that the action of MMP-2 has relevant physiopathological consequences on the coagulation process connected to the impairment of fibrin formation and the promotion of angiogenetic processes. Such a result is not necessarily in contradiction with data reported by Bini et al. [8], who claimed that, unlike for MMP-3, fibrinogen cleaved by MMP-2 showed a slight clot formation after several hours of processing by thrombin. This statement underlies a gross alteration of the clotting properties for the fibrinogen processed by MMP-2, in line with that observed in the present study for the fibrinogen cleaved by whole MMP-2 (Figure 1B).

It is interesting to observe that under the same experimental conditions of pH and temperature, plasmin is able to cleave all three chains of fibrinogen (Figure 1C) at a different rate (see Table 1). In addition, we have observed the formation of fragments which show a similar molecular mass to that observed in the case of MMP-2, suggesting that the cleavage of the intact chains by plasmin occurs at a site topologically close to that of MMP-2. However, unlike the enzymatic action by MMP-2, in the case of plasmin we also observed at short intermediate time intervals the formation of larger fragments (probably derived from the cleavage of α- and β-chains), which then disappeared as the presence of the smaller fragments increased (see Figure 1C), indicating that plasmin could have an additional more efficient cleavage site.

Furthermore, peroxynitrite-treated fibrinogen (already incapable of any clotting activity even in the presence of thrombin [18]), displayed a similar proteolytic processing by whole MMP-2 (Figure 1D), producing a similar fragment to that observed for

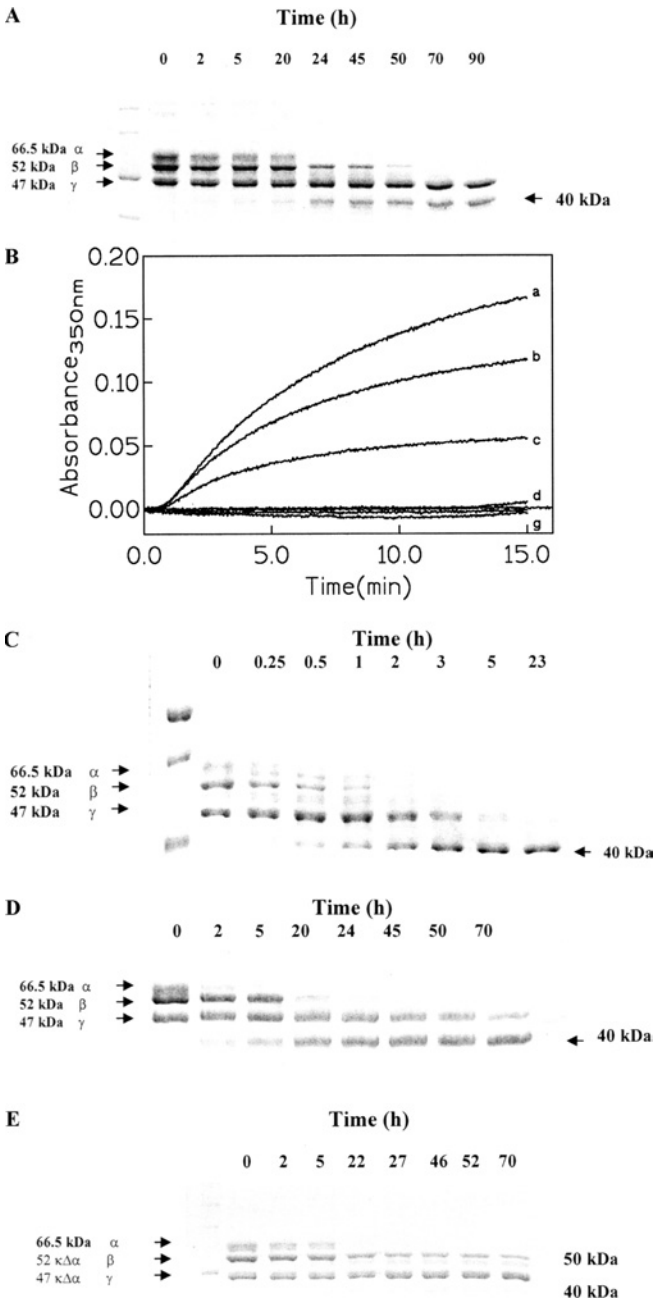

Figure 1 Kinetic proteolytic processing of bovine fibrinogen

(**A**) SDS/PAGE electrophoresis of the processing of bovine fibrinogen by whole MMP-2 as a function of time (as indicated) at 37 °C and pH 7.1. α- (66.5 kDa), β- (52 kDa) and γ- (47 kDa) chains are indicated as well as the 40 kDa fragment. (**B**) Clotting induced by human thrombin on bovine fibrinogen (curves a–c) and on bovine fibrinogen after degradation by whole MMP-2 (curves d–g). Fibrinogen was incubated at 37 °C in 50 mM Tris/HCl buffer (pH 8.2) and the clotting was followed at 350 nm after addition of 1.2 units of human thrombin. Curves a–c: 0.24, 0.16 and 0.08 mg/ml of bovine fibrinogen. Curves d–g: 0.08, 0.16, 0.24 and 0.32 mg/ml of fibrinogen degraded by whole MMP-2. (**C**) SDS/PAGE electrophoresis of the processing of bovine fibrinogen by plasmin as a function of time (as indicated) at 37 °C and pH 7.1. α- (66.5 kDa), β- (52 kDa) and γ- (47 kDa) chains are indicated as well as the 40 kDa fragment. (**D**) SDS/PAGE electrophoresis of the processing of peroxynitritre-treated bovine fibrinogen by MMP-2 as a function of time (as indicated) at 37 °C and pH 7.1. α- (66.5 kDa), β- (52 kDa) and γ- (47 kDa) chains are indicated as well as the 40 kDa fragment. (**E**) SDS/PAGE electrophoresis of the processing of bovine fibrinogen by the catalytic domain of MMP-2 as a function of time (as indicated) at 37 °C and pH 7.1. α- (66.5 kDa), β- (52 kDa) and γ- (47 kDa) chains are indicated as well as the 50 kDa and the 40 kDa fragments. For further details, see text.

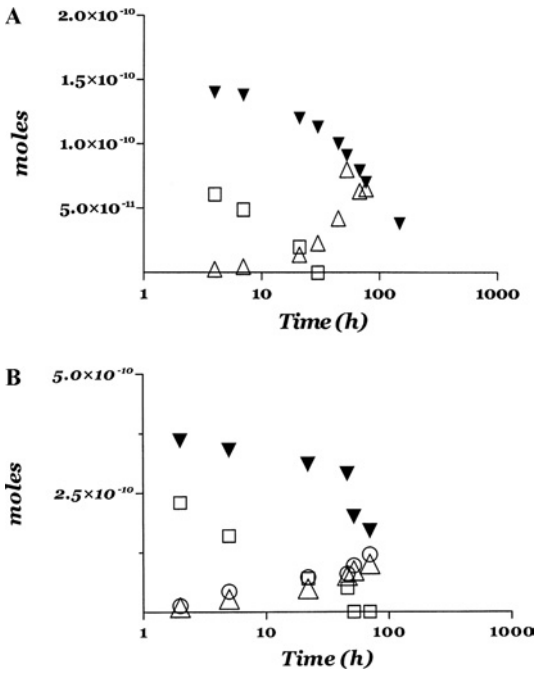

Figure 2 Temporal evolution of proteolytic processing of individual chains by MMP-2

(**A**) Temporal evolution of the α- (□) and β- (▼) chain degradation and of the 40 kDa fragment formation (△) during the processing of bovine fibrinogen by whole MMP-2. The amounts of each species are expressed in terms of the number of moles. (**B**) Temporal evolution of the α- (□) and β- (▼) chain degradation and of the 50 kDa (○) and 40 kDa (△) fragment formation during the processing of bovine fibrinogen by the catalytic domain of MMP-2. Amounts of each species are expressed in terms of number of moles. T = 37 °C and pH = 7.1. For further details, see text.

native fibrinogen, which suggested that the cleavage event(s) occur(s) at a similar site in native and in the peroxynitrite-treated fibrinogen. It is important to emphasize that from gradient electrophoresis observations no fragments of smaller molecular mass were observed (results not shown), ruling out the possibility of further fragmentation of the fibrinogen chains.

Degradation of fibrinogen by the catalytic domain of MMP-2

In order to clarify the role played by the haemopexin-like domain of MMP-2, we have also investigated the enzymatic processing of native fibrinogen by the catalytic domain of MMP-2. Figure 1(E) shows the time-dependent degradation of the three chains of fibrinogen by the catalytic domain of MMP-2 at pH 7.1 and 37 °C. It is immediately obvious that (i) the fragmentation occurs at a much lower rate with respect to the whole MMP-2 (Figures 1A and 2), and (ii) the proteolytic processing leads to the formation of a new fragment with a molecular mass intermediate between the β- and the γ-chain. This fragment looks similar to that observed during the early stages of the enzymatic processing of fibrinogen by plasmin (Figure 1C), but it is definitely not observed during the processing of native fibrinogen by whole MMP-2 (see Figures 1A and 1E). This difference becomes even more evident if we compare the time evolution of the increase in the different fragments with respect to the disappearance of the intact chains (Figure 2); thus, whereas in the case of whole MMP-2 the fragment(s) increase(s) concomitantly with the disappearance of the β-chain (Figure 2A), in the case of the fragment(s) formed by the catalytic domain of MMP-2 the time evolution of its appearance is

correlated to the disappearance of both α- and β-chains (Figure 2B). Together, these data seem to indicate that cleavage of the α-chains by whole MMP-2 does not produce enough fragments of a large enough size to be detected, whereas cleavage of β-chains by whole MMP-2 occurs at a preferential class of sites, with the production of sizeable fragments. Furthermore, the lack of the haemopexin-like domain, when we have only the catalytic domain (Figure 1E), brings about cleavage events at additional sites with respect to those involved in the fragmentation by the whole MMP-2. Taken together, these data indicate an important role by the haemopexin-like domain in the correct recognition process, as already observed for MMP-8 toward collagen I [30]. It must be emphasized that the clotting activity was also abolished in the case of fibrinogen processed by the catalytic domain of MMP-2 (results not shown), in a similar fashion to that observed for the fibrinogen processed by whole MMP-2 (Figure 1B). This clearly indicates an important pathophysiological role for MMP-2 in modulating the clotting activity of fibrinogen, independently of its molecular state.

In all cases (i.e. whole MMP-2 and catalytic domain with native and peroxynitrite-treated fibrinogen) the double reciprocal plot of the fibrinogen concentration and the enzymatic activity is linear for both chains of fibrinogen, clearly indicating that the first step occurs by a Michaelis–Menten mechanism (Figure 3). This consideration allows us to obtain the catalytic parameters for the cleavage of α- and β-chains of native fibrinogen by whole MMP-2 (Figure 3A) and its catalytic domain (Figure 3C), and by whole MMP-2 of fibrinogen treated with peroxynitrite (Figure 3B). These are reported in Table 1, showing that the lower enzymatic efficiency of the catalytic domain alone is due to a dramatically reduced kinetic constant for the rate-limiting cleavage step of the substrate.

Molecular modelling

In order to verify whether the functional data described above have a structural explanation, we have performed molecular modelling of the interaction between MMP-2 and fibrinogen. Given that MMP-2 is likely to be flexible due to the long linker connecting the haemopexin domain to the catalytic domain, it would not be fruitful to attempt to dock the X-ray structure of the complete enzyme with fibrinogen as a rigid body. Therefore for the simulations of the docking between whole MMP-2 and fibrinogen we have regenerated a 'flexible MMP-2', deleting the 20 amino acid residues of the linker and forcing the distance between the N-terminus of the catalytic domain and the C-terminus of the haemopexin domain to be no longer than 20 Å (1 Å = 0.1 nm) during the docking simulations. This provided us with the possibility of comparing the results for the two domains and, using constrained docking, we have attempted to simulate the mechanism by which the haemopexin-like domain may bring the catalytic domain into contact with fibrinogen at the correct region.

Figure 4(A) shows the results for the simulated docking of the catalytic domain (upper panel) and the haemopexin-like domain (lower panel) with fibrinogen. Both panels show 1000 models, with the domain of MMP-2 represented by a coloured sphere placed at its geometric centre in each model. The spheres are coloured according to the electrostatic interaction score, with red for the strongest (most negative) interactions, and green for the weakest interactions. We chose the electrostatic interaction score as the most representative of the interaction strength because it dominated strongly in the estimates given by BiGGER, with electrostatic energy being approx. 50 times stronger than the hydrophobic effects.

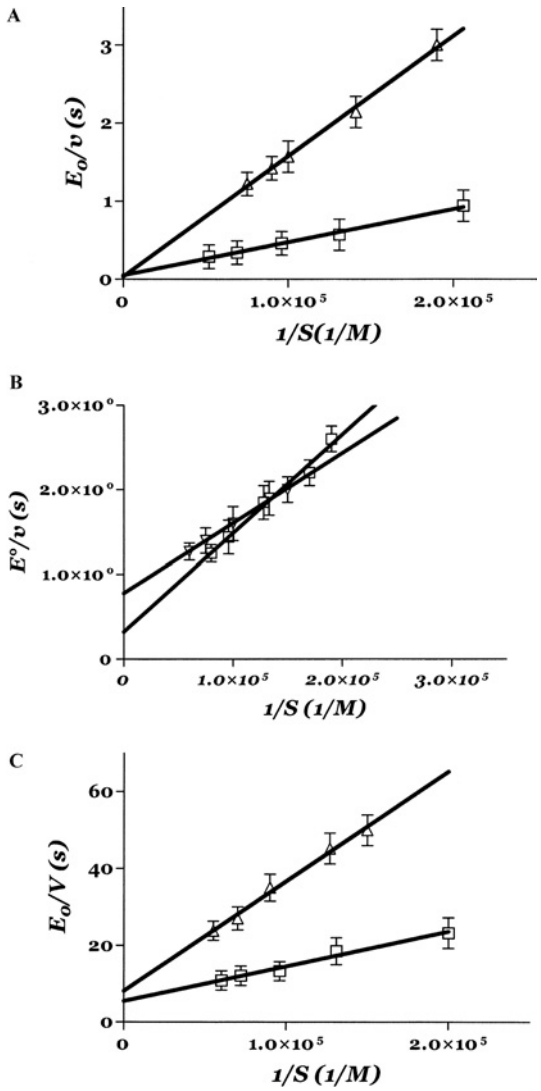

Figure 3 Lineweaver–Burk plots of the proteolytic processing of bovine fibrinogen chains by MMP-2

Lineweaver–Burk plots of the enzymatic processing by whole MMP-2 of α- (△) and β- (□) chains of native fibrinogen (**A**), peroxynitrite-treated fibrinogen (**B**) and of native fibrinogen by the catalytic domain of MMP-2 (**C**) at 37 °C and pH 7.1. Continuous lines have been obtained by non-linear least-squares fitting of data according to Eqn (1) and parameters are reported in Table 1. For further details, see text.

These results (Figure 4A) suggest that the binding of the haemopexin-like domain is more specific, since a large fraction of the models place the haemopexin-like domain near the C-terminal region of fibrinogen (to the left on Figures 4A and 4B), whereas the catalytic domain models are more evenly spread throughout the structure of fibrinogen. Not apparent in the Figure, but also important, is that the top electrostatic interaction score given by BiGGER is 50 % higher for the haemopexin-like domain than it is for the catalytic domain (−152 kCal/mol compared with −110 kCal/mol), suggesting that the interaction of the haemopexin-like domain is not only more specific but is also stronger.

The results shown in Figures 4(A) and 4(B) are for the docking of the active form of the catalytic domain, but we have also used the autoinhibitory procatalytic form of the catalytic domain, the catalytic domain lacking the fibronectin-like motif, and the fibronectin-like motif alone (results not shown). These simulations gave the results expected, producing neither a pattern

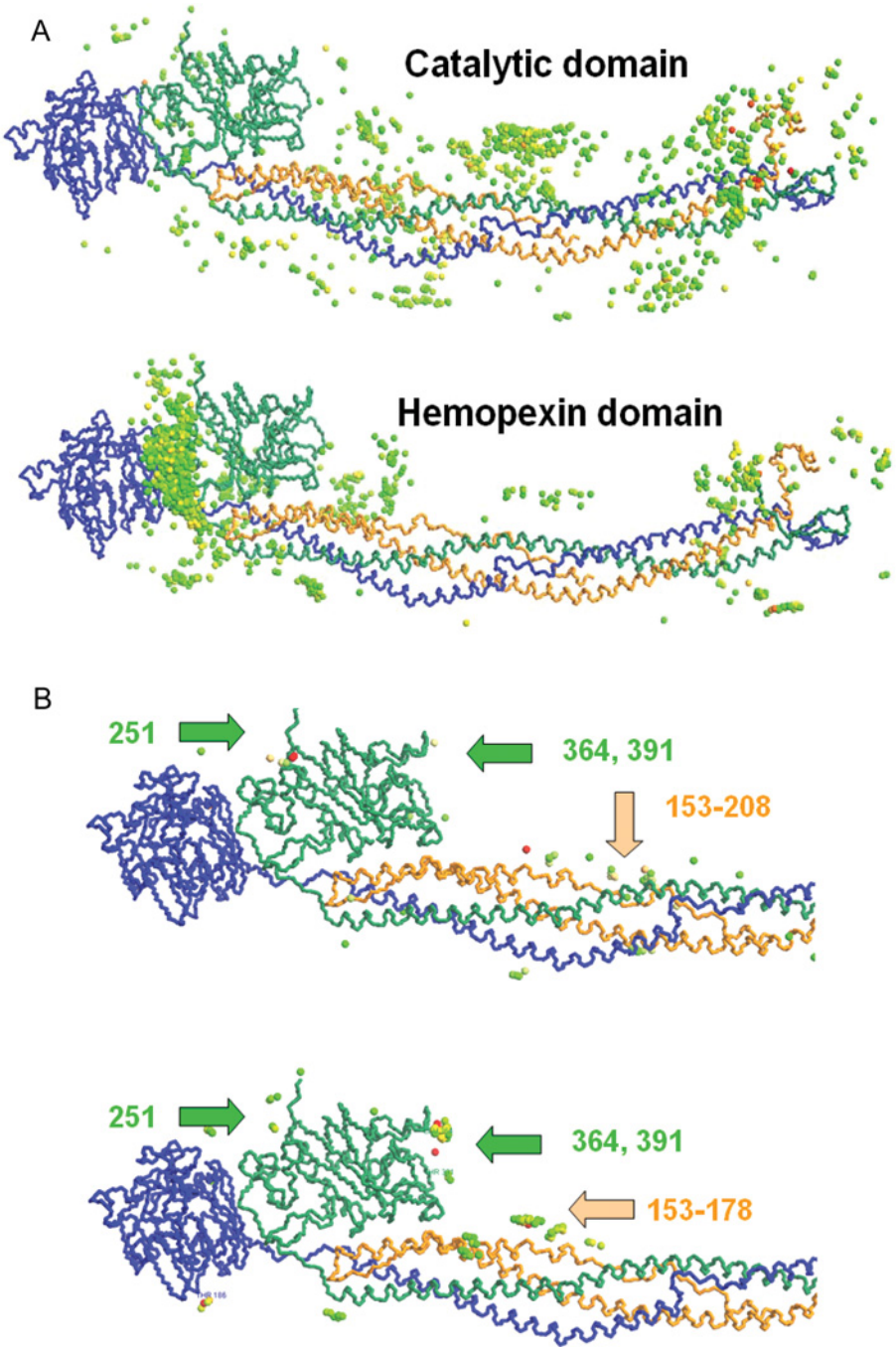

Figure 4 Molecular docking of MMP-2 on bovine fibrinogen

(**A**) Comparison of 1000 models for the docking of the catalytic domain (top panel) and the haemopexin-like domain (bottom panel) with fibrinogen. The fibrinogen monomer is shown at the centre of each panel (with α-, β- and γ-chains identified by orange, green and blue respectively), and each MMP-2 domain is represented by coloured spheres placed at their geometric centres. The models were selected and coloured according to the electrostatic interaction score, with red indicating stronger interactions and green weaker interactions. For the results reported in (**A**) no constraints were used, as each domain was docked independently to compare the specificity of their interactions. (**B**) Comparison of the regions of fibrinogen found to be accessible to the catalytic domain. Spheres represent the position of the catalytic Zn^{2+}. The top panel shows the models for the unconstrained docking of the catalytic domain; the bottom panel shows the models for the constrained docking of the catalytic domain with five complexes of the haemopexin-like domain and fibrinogen. Numbers indicate potential cleavage sites on the α- and β-chains of fibrinogen, as suggested by the docking of the complex between the catalytic domain and the haemopexin-like domain. For further details, see text.

of specific interactions nor interactions stronger than those for the active form of the catalytic domain of MMP-2. A closer examination of highest scoring models for the haemopexin-like domain docking indicated preferential binding sites which are grouped near the globular regions of the β-chains of the fibrinogen molecule. We selected the five highest ranking models from this simulation (according to the electrostatic score), and used the respective five complexes of fibrinogen with the haemopexin-like domain as targets for docking the catalytic domain of MMP-2. In addition, we imposed a constraint of 20 Å for the distance between the N-terminus of the haemopexin domain and the C-terminus of the catalytic domain, which corresponded to the

length of the linker segment we deleted when separating these two domains from the X-ray structure of the complete enzyme [31] (see above). Figure 4(B) (lower panel) shows the models from these five docking simulations in which the catalytic Zn^{2+} atom of the catalytic domain is within 5 Å of the backbone of fibrinogen. The justification for this selection was the assumption that the haemopexin-like domain is responsible for the specific and stable binding to the fibrinogen, placing the catalytic domain near the regions where the backbone of fibrinogen is accessible to the catalytic centre. Figure 4(B) shows the comparison of these constrained docking results with a similar selection (catalytic Zn^{2+} within 5 Å of the fibrinogen backbone) of the models obtained by docking the catalytic domain alone to the fibrinogen with no constraints.

These docking simulation results are in agreement with the experimental results obtained with the digestion of fibrinogen by MMP-2, which show that the haemopexin-like domain greatly increases the catalytic rate of the catalytic domain, and suggest that the catalytic domain alone cleaves fibrinogen with less specificity than the complete enzyme does, leading to the formation of additional fragments (Figure 1E). Comparing the unconstrained with the constrained docking simulations for the catalytic domain (Figure 4B) we observed a similar pattern of models where the catalytic Zn^{2+} can interact with the fibrinogen backbone, but with a lower specificity in the case of the unconstrained docking (upper panel), which showed a more disperse distribution of the catalytic domain. More significantly, the docking simulations indicated cleavage sites on the α- and β-chains only, represented in yellow and green respectively (Figure 4B), and none on the γ-chain, again in agreement with the experimental data obtained in the digestion kinetic experiments.

An analogous analysis has not been possible for the α-chain, since most of this chain is not visible from available crystallographic data due to a widespread disordered structural arrangement of this chain. As a matter of fact, docking simulations focussed on the available portion of the α-chain did not show any preferential interaction with whole MMP-2.

Effect of the haemopexin-like domain on the digestion of fibrinogen by whole MMP-2

In order to test the reliability of predictions from molecular modelling on the crucial role played by the haemopexin-like domain in the interaction between whole MMP-2 and the β-chain (and probably the α-chain) of fibrinogen (Figure 4), we performed fragmentation kinetics by whole MMP-2 of fibrinogen incubated at 37 °C with 200 μM haemopexin-like domain. Thus if the hypothesis suggested by the molecular modelling is correct, at such a concentration the haemopexin-like domain should almost fully occupy the binding site, severely impairing the fragmentation of fibrinogen by whole MMP-2, as suggested by the K_m value (Table 1). Figure 5 reports the outcome of this experiment performed in parallel with the fragmentation of fibrinogen alone, clearly showing that the presence of a 200 μM haemopexin-like domain greatly inhibits the fragmentation of the α- and β-chains of fibrinogen. This is a very important result since it (i) directly demonstrates the validity of the hypothesis formulated on the basis of molecular modelling, allowing us to propose the interaction of the haemopexin-like domain with the β-chain of fibrinogen as an actual mechanism and not simply as a working hypothesis; and (ii) rules out the possibility that other contaminating MMPs contribute significantly to the observed phenomenon, since the haemopexin-like domain can only interfere with intact MMP-2 and its reaction with fibrinogen is shown to essentially abolish the proteolytic cleavage.

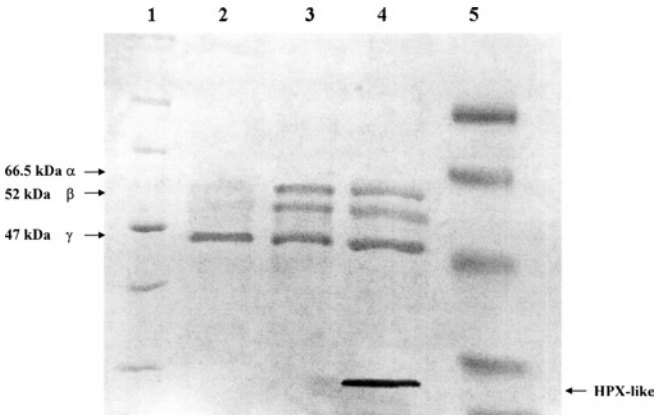

Figure 5 Inhibitory effect of the haemopexin-like domain on the proteolytic processing of fibrinogen by MMP-2

SDS/PAGE electrophoresis of fibrinogen degradation by whole MMP-2 after 20 h at 37 °C in the absence (lane 2) and in the presence of 200 μM haemopexin-like domain (lane 4). Lane 3 shows the intact fibrinogen. Lane 1 shows the the low molecular mass markers and lane 5 shows the broad molecular mass markers. Chains of fibrinogen and the haemopexin-like domain are indicated by arrows. For further details, see text.

The pH-dependence of catalytic parameters for MMP-2 on fibrinogen

The pH-dependence of catalytic parameters for MMP-2 on the α- and β-chains of native fibrinogen has been characterized and the values of pK_{aU} (corresponding to values in the free enzyme) and pK_{aL} (corresponding to values in the substrate-bound enzyme) have been determined on the basis of the pH-dependence of different catalytic parameters according to the following equations [26]

$$^{obs}\left(\frac{k_{cat}}{K_m}\right) = \frac{\sum_{i=0}^{i=n} \frac{k_{cati}}{K_{mi}} \prod_{r=0}^{r=i} K_{aUr} \cdot [H^+]}{\sum_{i=0}^{i=n} \prod_{r=0}^{r=i} K_{aUr} \cdot [H^+]} \quad (2)$$

$$^{obs}k_{cat} = \frac{\sum_{i=0}^{i=n} k_{cati} \prod_{r=0}^{r=i} K_{aLr} \cdot [H^+]}{\sum_{i=0}^{i=n} \prod_{r=0}^{r=i} K_{aLr} \cdot [H^+]} \quad (3)$$

$$^{obs}K_m = K_{m0} \cdot \frac{\sum_{i=0}^{i=n} \prod_{r=0}^{r=i} K_{aUr} \cdot [H^+]}{\sum_{i=0}^{i=n} \prod_{r=0}^{r=i} K_{aLr} \cdot [H^+]} \quad (4)$$

where $^{obs}(k_{cat}/K_m)$, $^{obs}k_{cat}$ and $^{obs}K_m$ are the values of these catalytic parameters at a given pH, i $(=0,1,\ldots n)$ are the different protonation states [with $(k_{cat}/K_m)_0$, k_{cat0} and K_{m0} the values of the catalytic parameters in the unprotonated form], K_{aUi} $(=10^{pKaUi})$ and K_{aLi} $(=10^{pKaLi})$ are the ith proton-binding constants for the free reactants and the enzyme–substrate complex respectively, and $[H^+]$ is the proton concentration. Experimental data have been fitted with Eqn 2–4, employing $n=3$ and this proton-linked modulation is described in Figure 6 with parameters reported in Table 2.

The data in Table 2 make it very clear that the pH-dependence profile of the overall enzymatic activity (i.e. k_{cat}/K_m) is similar for the two chains and quite complex (Figure 6A), requiring the involvement of (at least) three protonating groups. On the other

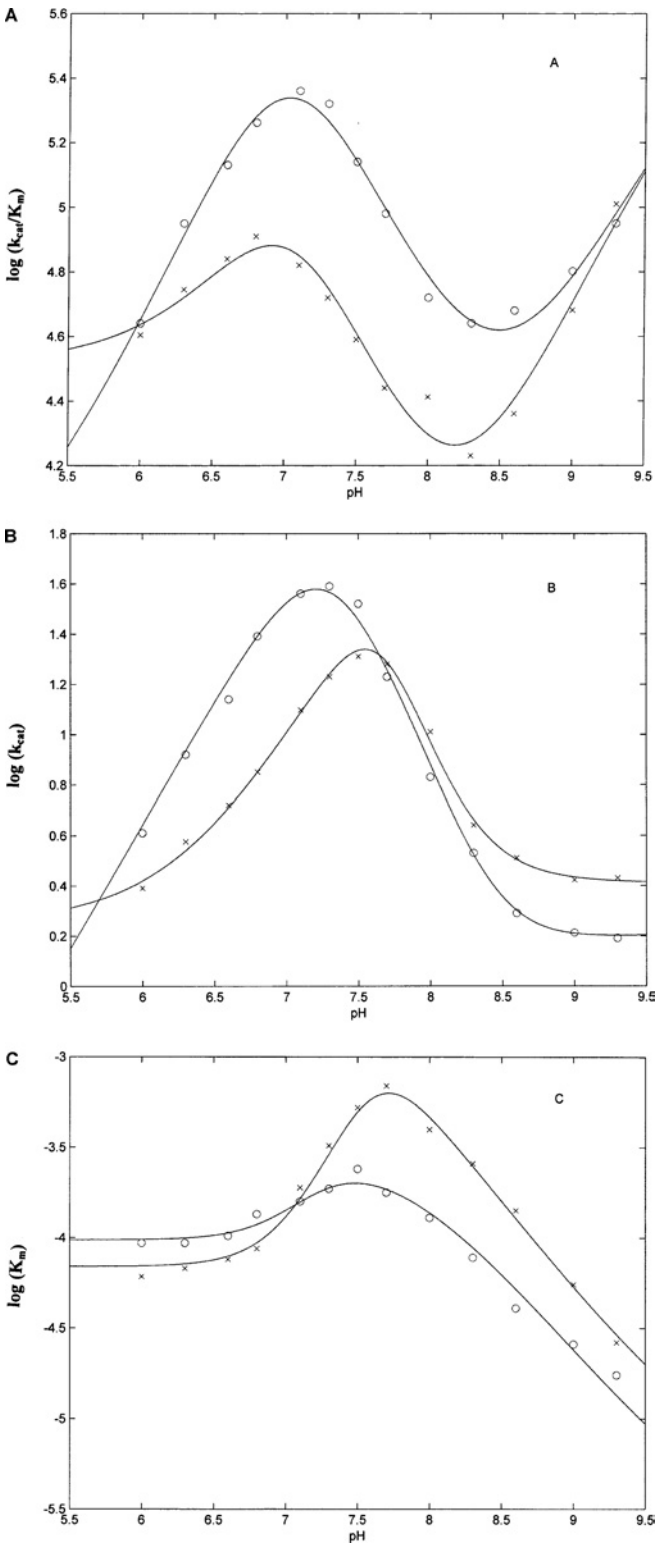

Figure 6 pH dependence of k_{cat}/K_m (A), k_{cat} (B) and K_m (C) for the processing of α- (○) and β- (x) chains of bovine fibrinogen by whole MMP-2 at 37 °C

Continuous lines correspond to non-linear least-squares fitting of data according to Eqns 2, 3 and 4, and values of pK_{aU} and pK_{aL} for the two chains are reported in Table 2. For further details, see text.

hand, the pH-dependence of the rate-limiting step (i.e. k_{cat}) shows a somewhat simpler bell-shaped profile and it appears to be slightly displaced to more alkaline pH values in the case of the β-chain

Table 2 Values of pK_{aU} and pK_{aL} for the pH dependence of enzymatic processing of α- and β-chains of fibrinogen by whole MMP-2 at 37 °C

	α-Chain	β-Chain
pK_{aU1}	10.04 ± 0.15	10.04 ± 0.17
pK_{aU2}	4.07 ± 0.16	4.07 ± 0.15
pK_{aU3}	9.97 ± 0.17	9.97 ± 0.18
pK_{aL1}	7.86 ± 0.16	4.86 ± 0.17
pK_{aL2}	4.52 ± 0.16	7.72 ± 0.15
pK_{aL3}	10.05 ± 0.17	10.32 ± 0.17

(Figure 6B). As a consequence, a similar displacement is observed for K_m (Figure 6C), which is affected only by those groups whose pK_a values change upon substrate binding (see Table 2).

It is important to remark that resulting pK_a values are different from those reported previously for MMP-2 against synthetic substrates [26]; this is quite obvious, since different residues are involved in the two cases, as indicated by the fact that MMP-2 interacts through the haemopexin-like domain with fibrinogen, whereas the binding of synthetic substrates only involves residues in the immediate vicinity of the catalytic site. Obviously, we cannot rule out the possibility that some of these pK_a values also refer to residue(s) of fibrinogen, which change their value upon interaction with MMP-2.

From an overall view of the pH-dependence of these three catalytic parameters it emerges that the decrease of k_{cat}/K_m between pH 9.5 and pH 8.5 (Figure 6A) is due to a marked increase of K_m (that is a decrease of substrate affinity) over the same pH range (Figure 6C), which is only modestly compensated by a small increase of k_{cat} (Figure 6B). On the other hand, the enhancement of k_{cat}/K_m between pH 8.5 and pH 7.0 (Figure 6A) is mostly due to the increase of k_{cat}, which attains the maximum value at pH 7.2 for the α-chain and at pH 7.5 for the β-chain (Figure 6B). Over the same range, K_m is providing only a minor contribution, since it attains the minimum affinity value at pH 7.4 for the α-chain and at pH 7.8 for the β-chain (Figure 6C). The decrease of k_{cat}/K_m at pH < 7.0 (Figure 6A) is again induced by the decrease of the rate-limiting step k_{cat} (Figure 6B), which overwhelms the increasing affinity of MMP-2 for the substrate (as indicated by the decrease of K_m; Figure 6C). Therefore we can conclude that the proteolytic activity of MMP-2 towards the two chains of fibrinogen is dominated at alkaline pH values by the substrate affinity, whereas as the pH is lowered the catalytic efficiency becomes dominated by the velocity of the rate-limiting step.

DISCUSSION

The role of MMP-2 in the neovascularization process is very well established and so is its capability to enzymatically process fibrinogen and cross-linked fibrin [8]. A very important aspect of the enzymatic action of MMP-2 on fibrinogen is the observation that the cleavage brings about an impairment in the formation of fibrin clots upon exposure to the action of thrombin (Figure 1B). It clearly means that this process induces a gross alteration in the coagulation process with important pathological consequences. Therefore since MMP-2 and fibrinogen are coming into close contact in the bloodstream, a kinetic analysis of the proteolytic processing by whole MMP-2 of native fibrinogen and of peroxynitrite-treated fibrinogen (a condition present under oxidative stress and which is known to impair the formation of clots [18]) is of the utmost importance for the comprehension of such a relevant event. This is clearly demonstrated by the fact that the catalytic efficiency of MMP-2 on fibrinogen (as indicated

by k_{cat}/K_m) is somewhat lower than that of thrombin [20] and similar to that displayed by plasmin [21]. In addition, in order to uncover the determinants of the enzyme–substrate recognition process, the enzymatic inactivation of native fibrinogen by the catalytic domain of MMP-2 and by intact MMP-2 in the presence of a saturating amount of haemopexin-like domain has been investigated, allowing the role of the haemopexin-like domain in this relevant process to be unequivocally established. Furthermore, the pH-dependence of catalytic parameters for the processing of both fibrinogen chains by whole MMP-2 allows the substrate-binding mechanism and the modulation of enzymatic activity by environmental conditions to be clarified.

Table 1 reports the catalytic parameters for this process at pH 7.1 and 37 °C, which indicates that the overall enzymatic activity, as indicated by values of k_{cat}/K_m, of intact MMP-2 is faster on the α-chain as compared with the β-chain of native fibrinogen (mostly due to a higher k_{cat}; see Table 1), while it is closely similar for the two chains in the peroxynitrite-treated fibrinogen. Furthermore, the removal of the haemopexin-like domain brings about a dramatic 20-fold reduction in the enzymatic activity with respect to the intact MMP-2, though keeping the approx. 3-fold preferential action for the α-chain (mostly due to a difference in K_m; see Table 1), suggesting a difference for the substrate-recognition process according to whether MMP-2 contains the haemopexin-like domain or not. This is in accordance with a variation in the binding mode between the whole MMP-2 and the catalytic domain. Thus in the first case binding is driven by the haemopexin-like domain (which has a similar affinity for the two chains, as suggested by the similar K_m of intact MMP-2; Table 1), whereas in the second case the process is regulated by the interaction of the catalytic domain (which interacts differently with the cleavage site of the two chains, as suggested by the different k_{cat} in the whole MMP-2; Table 1). This result, which is supported by data on collagen reported by others [32,33], would suggest that MMP-2 interacts with fibrinogen by first binding through the haemopexin domain, which then acts as an anchor to force the catalytic domain into contact with the substrate. The occurrence of this interaction, which would also be in agreement with previous data indicating that binding of the haemopexin-like domain is driven by electrostatic forces [34], is demonstrated unequivocally by the experiment shown in Figure 5, which shows that in the presence of the haemopexin-like domain the degradation of fibrinogen by whole MMP-2 is severely impaired. As a whole, at pH 7.1 a picture emerges in which whole MMP-2 binds the α- and β-chains of native fibrinogen with equal probability (as seen from the closely similar values of K_m; Table 1) by means of the haemopexin-like domain (Figure 4). Moreover, data reported in Table 1 indicate that MMP-2 cleaves the two chains at a different rate, mostly because the catalytic domain appears to preferentially process the α-chain, which is probably more exposed.

It is interesting to observe that comparing the behaviour of intact MMP-2 with plasmin shows a drastic difference in that plasmin is also able to enzymatically process the γ-chain of fibrinogen (Figure 1C), whereas intact MMP-2 does not. Furthermore, observing the catalytic parameters reported in Table 1, it emerges that the catalytic efficiency (as expressed by k_{cat}/K_m) of intact MMP-2 is closely similar to that of plasmin on the α-chain of fibrinogen, as a result of a counterbalancing between the velocity of the rate-limiting step (as expressed by k_{cat}), which is higher for intact MMP-2 and the substrate affinity (as expressed by K_m), which is higher for plasmin. On the other hand, plasmin shows a 4-fold better activity with respect to intact MMP-2 on the β-chain of fibrinogen, which is almost fully attributable to a faster k_{cat} (Table 1). As a whole, it seems that the enzymatic action of plasmin and MMP-2 are fairly similar with the exception of the fact that plasmin is able to cleave the γ-chain, probably reflecting the smaller size of the enzyme and its higher flexibility.

Interpretation of data obtained for the catalytic domain of MMP-2 on native fibrinogen appears somewhat puzzling compared with that of intact MMP-2. Thus the removal of the haemopexin-like domain seems to bring about a dramatic decrease (by two orders of magnitude) for the rate constant of the rate-limiting step (i.e. k_{cat}), partially compensated by an increased affinity by about one order of magnitude for both chains (as seen from the decrease of K_m; Table 1). At first sight, this result seems to contradict the relevance of the role played by the haemopexin-like domain in the substrate recognition, since, this being the case, we would have expected a dramatic decrease of affinity, and thus an increase of K_m, upon removal of the haemopexin-like domain. Actually, these data can be somehow reconciled with the picture given above for the whole MMP-2, suggesting a much more complex role of the haemopexin-like domain. Thus the drastically different value of k_{cat} underlies a 'correct' substrate recognition (such that MMP-2 is able to cleave fibrinogen at the correct place) only in the presence of the haemopexin-like domain, suggesting an 'unproductive' enzyme–substrate complex in its absence, as also observed for collagen I [25,30,31,35]. Therefore the decrease of K_m (Table 1) for the catalytic domain may be attributed to the appearance, consequent to the removal of the haemopexin-like domain, of a new interaction site between MMP-2 and fibrinogen, with the consequent formation of new fragments (Figures 1E and 2B) at a much less efficient rate.

A different effect must come into play in the cleavage mechanism of peroxynitrite-treated fibrinogen by whole MMP-2. In this case, it appears as if the peroxynitrite-induced oxidation of fibrinogen brings about a structural alteration of the molecule, which renders it more easily recognizable by MMP-2 (as seen by the lower value of K_m; Table 1), but more resistant to the cleavage action (as seen by the lower value of k_{cat}; Table 1). The net result is a similar overall enzymatic action by MMP-2 with respect to native fibrinogen (as seen by the similar value of k_{cat}/K_m, Table 1; and the same type of fragment produced by the cleavage action, Figure 1). This conformational change occurring in peroxynitrite-treated fibrinogen is also probably responsible for the disappearance of catalytic heterogeneity of the two chains. It makes the interaction of MMP-2 with both chains of fibrinogen much stronger (this feature being particularly evident for the β chain), but it also leads to a drastically reduced susceptibility of both chains to the proteolytic cleavage (the effect being especially pronounced for the β chain; Table 1).

An interesting aspect of catalytic parameters for the activity of intact MMP-2 on native fibrinogen is the close similarity of K_m for both α- and β-chains (Table 1). The whole MMP-2 has the same affinity for α- and β-chains, suggesting the possibility that there is only one binding site for MMP-2 on fibrinogen. If this were true, the binding site could be located near the globular D-domain of the β-chain, as suggested above by the molecular modelling (Figure 4) and by the experiment in the presence of the haemopexin-like domain (Figure 5), and the same bound molecule could alternatively cleave either the α- and/or β-chains.

In order to discriminate between the possibility of a single binding site and that in which fibrinogen has two different binding sites for MMP-2 (one on the α-chain and one on the β-chain), we have investigated the pH-dependence of catalytic parameters for MMP-2 on the α- and β-chains of native fibrinogen. Thus following this approach the occurrence of a single binding site in fibrinogen for MMP-2 demands that the pH-dependence for K_m is the same in the case of the α- and β-chain. This possibility is ruled out by the different pH-dependence of K_m for the two chains (Figure 6C), unequivocally demonstrating that in fibrinogen there

are two independent binding sites for MMP-2 (one at the α-chain and one at the β-chain).

A closer look at parameters reported in Table 2 shows that all three pK_{aU} values are the same for both fibrinogen chains, indicating that the same groups of MMP-2 are involved in the proton-linked modulation of catalytic properties toward the two chains. This is also an indirect indication that two different MMP-2 molecules interact with the two chains, even though the interaction mode must differ for the two chains, as seen by the different pK_a shifts (resulting in different pK_{aL} values; Table 2).

The identification of groups involved in this proton-linked modulation of the enzymatic activity of MMP-2 towards fibrinogen is quite difficult, since an accurate identification would require an extensive study employing site-directed mutants. On the other hand, we cannot rule out the possibility that some of the pK_a values refer to residue(s) of fibrinogen, which change their values upon interaction with MMP-2. However, some tentative attribution can be undertaken based on the pK_a values and on their shift, taking into consideration the fact that not all three groups must necessarily be in close proximity to the active site.

In this respect, the residue responsible for pK_{a1}, which displays a fairly high pK_{aU} value (possibly referring to a tyrosine residue) before the enzyme–fibrinogen interaction, undergoes a drastic shift with a proton release upon substrate binding, much more pronounced for the interaction of MMP-2 with the β-chain (Table 2). This behaviour suggests that in the enzyme–substrate complex the environment is very polar and positively charged, with a consequent marked enhancement of its acidic character, which might render its protonation down to fairly low pH values very difficult.

On the other hand, the residue responsible for pK_{a2}, which shows a quite low value for pK_{aU} before the interaction (possibly referring to a histidine residue), changes very little upon the interaction of MMP-2 with the α-chain, but it is upshifted to a much larger extent when the enzyme interacts with the β-chain (Table 2). The different behaviour for the two chains suggests that this residue might be located in a region of MMP-2 which interacts with two drastically different regions of the two chains. Thus, unlike what is observed in the case of the residue responsible for pK_{a1} (see above), this residue weakens its acidic character taking up a proton upon interaction with the β-chain, while it is very marginally affected by the interaction with the α-chain.

In the case of the residue responsible for the pK_{a3}, it displays a very marginal pK_a increase upon substrate binding (by a similar extent for the two chains; Table 2), suggesting only a modest change in the environment upon substrate binding for both chains.

In conclusion, from the present study several novel aspects of this relevant process emerge, such that (i) different domains of MMP-2 contribute in a synergistic way to the proteolytic processing of fibrinogen; (ii) this proteolytic cleavage brings about the formation of an inactive form of fibrinogen, thus grossly affecting the coagulation process; (iii) MMP-2 binds with the haemopexin-like domain, enzymatically processing both α- and β-chains, whereas there is no evidence for the cleavage of the γ-chain; (iv) the peroxynitrite-treated fibrinogen undergoes a conformational change which affects the enzymatic mechanism by which MMP-2 processes fibrinogen; and (v) the pH-dependence of catalytic parameters for MMP-2 on fibrinogen required at least three protonating groups whose pK_a values change upon substrate binding.

A final comment must emphasize that inactivation of fibrinogen by both whole MMP-2 and the catalytic domain of MMP-2 is of great pathophysiological relevance in view of the values of the catalytic parameters, not dramatically different from those observed for thrombin and plasmin [20,21] and for the important role played by MMP-2 in promoting inflammation and in favouring angiogenesis [7–9]. These data also give functional support to the crucial role played by MMP-2 in inducing vascular and circulatory alterations under chronic inflammatory conditions, such as those occurring in atherosclerosis and diabetes [36,37].

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Enzymatic processing of collagen IV by MMP-2 (gelatinase A) affects neutrophil migration and it is modulated by extracatalytic domains

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Abstract

AU1 Proteolytic degradation of basement membrane influences the cell behavior during important processes, such as inflammations, tumorigenesis, angiogenesis, and allergic diseases. In this study, we have investigated the action of gelatinase A (MMP-2) on collagen IV, the major constituent of the basement membrane. We have compared quantitatively its action on the soluble forms of collagen IV extracted with or without pepsin (from human placenta and from Engelbreth–Holm–Swarm [EHS] murine sarcoma, respectively). The catalytic efficiency of MMP-2 is dramatically reduced in the case of the EHS murine sarcoma with respect to the human placenta, probably due to the much tighter packing of the network which renders very slow the speed of the rate-limiting step. We have also enquired on the role of MMP-2 domains in processing collagen IV. Addition of the isolated collagen binding domain, corresponding to the fibronectin-like domain of whole MMP-2, greatly inhibits the cleavage process, demonstrating that MMP-2 interacts with collagen type IV preferentially through its fibronectin-like domain. Conversely, the removal of the hemopexin-like domain, using only the catalytic domain of MMP-2, has only a limited effect on the catalytic efficiency toward collagen IV, indicating that the missing domain does not have great relevance for the overall mechanism. Finally, we have investigated the effect of MMP-2 proteolytic activity ex vivo. MMP-2 action negatively affects the neutrophils' migration across type IV coated membranes and this is likely related to the production of lower molecular weight fragments that impair the cellular migration.

Keywords: endopeptidase/gelatinase A; collagen IV; fragmentation; kinetics; cell migration

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Abbreviations: EHS, Engelbreth–Holm–Swarm murine sarcoma; rCBD, recombinant collagen binding domain from MMP-2; cdMMP-2, catalytic domain of MMP-2; LPS, lipopolysaccharide; OPD, orthophenilendiamine.

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Gelatinases (MMP-2, MMP-9), like the other matrix metalloproteinases (MMPs), are a class of Ca^{+2}- and Zn^{+2}-dependent endopeptidases characterized by a Zn^{+2} atom on the active site that is coordinated by three histidyl residues. They show a multidomain structural organization, usually made by a propeptide domain (which is removed upon enzyme activation), the catalytic domain, and a hemopexin-like domain, which are connected by a hinge domain

(Sternlicht and Werb 2001). In addition, the two matrix metalloproteinases MMP-2 and MMP-9 possess a unique collagen binding domain (CBD), called the fibronectin-like domain, inserted on the catalytic domain and consisting of three 58–amino acid fibronectin type II-like modules (Allan et al. 1995). Furthermore, the membrane-bound MMPs have an intramembrane portion with a short cytoplasmatic tail. These ancillary exosites play an important role in the biochemistry of different substrates' recognition, and a great amount of information has been built up in the past years to elucidate the modulation mechanism of this crucial aspect (Marini et al. 2000; Patterson et al. 2001; Gioia et al. 2002; Overall 2002; Chung et al. 2004; Tam et al. 2004; Xu et al. 2005).

Like other MMPs, gelatinases cleave most of the major macromolecules of the extracellular matrix (i.e., collagen types IV, V, VII, and X; elastin; and others) and activate growth factors and chemokines, thus participating in normal tissue homeostasis and different kind of pathologies, such as arthritis (Dean et al. 1989; Somerville et al. 2003). In particular, it has been shown that MMP-2 and MMP-9 are involved in the so-called "angiogenic switch," a process consisting of the induction of a new vasculature during tumor dissemination and wound healing (Bergers et al. 2000; Fang et al. 2000). In fact, during the degradation of type IV collagen, a major component of the vascular basement membrane, gelatinases lead to VEGF release and the consequent migration and proliferation of the endothelial cells in the provisional matrix in order to create the new blood vessels (Kalluri 2003). Moreover, processing of type IV collagen gives rise to the release of fragments located in the collagenous domain that are able to behave as epitopes, since they can be bound by circulating IgA antibodies. This is the case of recurrent Goodpasture's disease secondary to an autoreactive IgA antibody (Borza et al. 2005).

Collagen type IV represents ~50% of all basement membrane proteins, and it is also termed "network-forming collagen" for its capacity to self-assemble into organized networks. In fact, unlike fibrillar collagens of types I, II, III, and V, type IV collagen forms a network structure and it is found crucial for basement membrane stability and assembly, as demonstrated in murine Engelbreth–Holm–Swarm sarcoma (EHS) (Kühn et al. 1981; Timpl et al. 1981). Type IV collagen in mammals is derived from six genetically distinct α-chain polypeptides (serially numbered from α-1 to α-6), with similar domain structures, which share a 50%–70% homology at the amino acid level; the different chains differ from one another in length, being distinct from those forming fibrillar collagens for imperfections in the triple helix of the collagenous domain. The α-chains can be separated into three domains: an amino-terminal 7S domain, a middle triple-helical domain, and a carboxy-terminal globular noncollagenous domain (called NC1). The NC1 domain is considered important for the assembly of the trimeric structure, which is made by three α-chains associated through their NC1 terminal domain to form a trimer called protomer, and in this respect a role of driving force can be proposed for the α-2 chains (Khoshnoodi et al. 2006). The protomer is formed by three α-chains, usually two α-1 chains and one α-2 chain, whose primary structures indicate an apparent molecular weight of ~185 and 170 kDa, respectively (Hostikka and Tryggvason 1987; Soininen et al. 1987). Further, two protomers of type IV collagen associate via NC1 trimers to give an NC1 hexamer, and finally four hexamers interact at the glycosylated diamino-terminal 7S region to form tetramers. This structure is the nucleus of the type IV collagen scaffold, which assembles in a suprastructure with laminin polymers during basement membrane formation (Kalluri 2003) and it begins to unfold only at relatively high temperatures, namely, over 40°C (Dölz et al. 1988). The degradation of type IV collagen is a very important process, since it occurs during several physiological and pathological processes involving the basement membrane, such as embryonic development or tumorigenesis (Ortega and Werb 2002), producing the exposure of cryptic sites important for angiogenesis (Xu et al. 2001). However, it has been also shown recently that cleavage of collagen IV, and particularly of its noncollagenous domains, brings about the production of lower molecular weight fragments, which inhibit angiogenesis and tumor growth (Petitclerc et al. 2000; Roth et al. 2005), envisaging the possibility of multiple actions with opposite effects connected to the enzymatic processing of collagen IV by MMPs.

Cell migration (and in particular neutrophil migration) across the basement membrane is a relevant process (in which collagen IV is certainly involved) during the acute phases of inflammation and allergic diseases (Tani et al. 2001). Such a phenomenon has been heavily associated to the proteolytic activity of MMP-9 and elastase, which are the main components of neutrophil secretion able to cleave collagen IV (Delclaux et al. 1996). However, they cannot be the only proteolytic enzymes involved in the migration across the basement membrane, since mice knockout for MMP-9 display as well a migration capability (Betsuyaku et al. 1999).

Therefore, since previous papers have already shown the capability of MMP-2 to process type IV collagen from different sources (Mackay et al. 1990; Eble et al. 1996) and the fragment originating from collagen IV degradation has been shown to play a relevant role in the modulation of several processes, such as migration and apoptosis (Ortega and Werb 2002), we decided to investigate the possible auxiliary role of MMP-2 on neutrophil migration. In this article we associate this role to a quantitative analysis of the catalytic efficiency of MMP-2 on type IV collagen from commercial human placenta (pretreated with pepsin), as

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well as on native type IV collagen from EHS, in order to characterize some aspects of the mechanism by which gelatinase A proteolytically processes collagen IV. These two types of collagen IV sources have been selected because they are the most commonly employed in the literature and they are representative of a type IV collagen partially digested (i.e., that from human placenta) and of a substantially intact collagen IV (i.e., that from murine EHS sarcoma). This parallel approach, never carried out before, allows us to fully characterize for the first time the catalytic parameters of MMP-2 for different subunits of both type IV collagens. It is very important to note that this overall analysis (from the molecular and cellular standpoint) allows us to relate the activity of MMP-2 on collagen IV to its negative role on the migration of neutrophils across a membrane coated with both sources of type IV collagen. This behavior is likely related to the production of lower molecular weight fragments, which impair the migration process (Petitclerc et al. 2000; Roth et al. 2005), and such an investigation represents a first attempt to obtain a deeper insight into a functionally relevant mechanism, in spite of the unavoidable limitations to the extrapolation to in vivo conditions.

Results

Intact MMP-2 and its catalytic domain alone (i.e., lacking the hemopexin-like domain) display a closely similar electrophoretic pattern for the degradation of type IV collagen from human placenta, acting on the same species but with different catalytic parameters (see below). In F1 Figure 1A we report a typical electrophoretic pattern at 37°C and at pH 7.3, where it is possible to observe that only three species are progressively cleaved by MMP-2—namely, those characterized by molecular masses of 207, 169, and 92 kDa, respectively—whereas the other species seem not to be affected by the cleavage event.

Considering that the structure of type IV of collagen is very complex, it is not so easy to identify the different species involved in the final structure. For the three species enzymatically processed by MMP-2, the 92-kDa species turns out to be a fragment of the α-2 chain (P. van den Steen and G. Opdenakker, pers. comm.), resulting from the pepsin digestion and likely not involved in the triple helix and partially unwound. On the other hand, the 207- and the 169-kDa species are likely referable to the intact α1-chain and α2-chain, respectively, on the basis of sequence-based mass determination (Hostikka and Tryggvason 1987; Soininen et al. 1987).

It is important to underline that the first cleavage step of the enzymatic processing appears to follow the Michaelis–Menten mechanism for all three species, as from the linearity of the reciprocal plot for the velocity versus substrate concentration (see Fig. 1B–D).

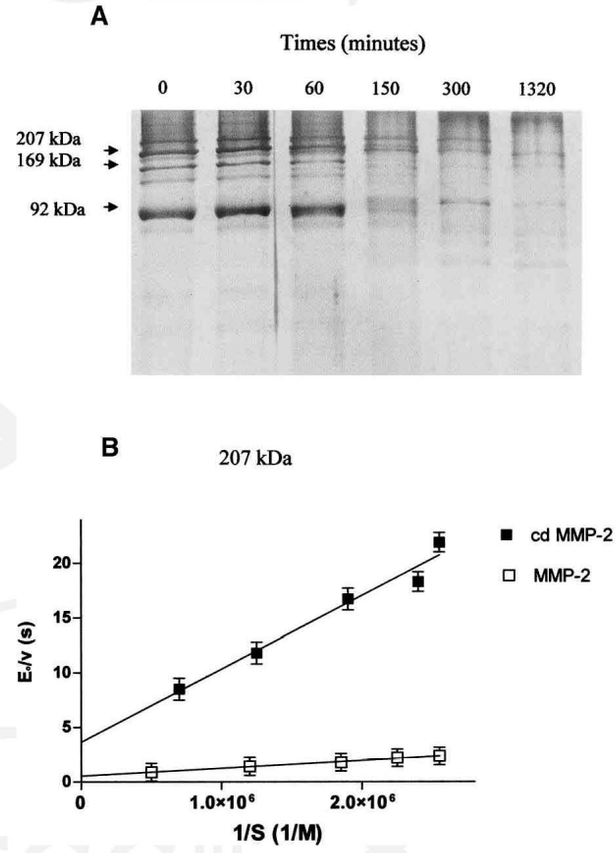

Figure 1. Enzymatic processing of collagen IV from human placenta by MMP-2 and double-reciprocal plots of the enzymatic processing of different chains by whole MMP-2 and by the catalytic domain of MMP-2. (*A*) SDS-PAGE electrophoresis of the processing of collagen type IV from human placenta by the catalytic domain of MMP-2 as a function of time (0, 30, 60, 150, 300, and 1320 min) at 37°C and pH 7.3. Fragments under investigation are indicated by their molecular mass: 207, 169, and 92 kDa. (*B*) Lineweaver–Burk plots of the processing by whole MMP-2 (□) and by the catalytic domain of MMP-2 (■) of the 207-kDa fragment. (*C*) Lineweaver–Burk plots of the processing by whole MMP-2 (□) and by the catalytic domain of MMP-2 (■) of the 169-kDa fragment. (*D*) Lineweaver–Burk plots of the processing by whole MMP-2 (□) and by the catalytic domain of MMP-2 (■) of the 92-kDa fragment. (For further details, see text.)

Degradation of collagen type IV from human placenta by whole MMP-2

The first degradation step was characterized by catalytic parameters reported in Table 1, where intact MMP-2 turns T1 out to cleave the two putative α-1 (207 kDa) and α-2 chains (169 kDa) with a similar catalytic efficiency (as represented by k_{cat}/K_m), while the processing of the 92-kDa species takes place with an efficiency lower by 1 order of magnitude. However, if we dissect the overall enzymatic activity, splitting the contribution arising from the substrate recognition (as grossly referable to K_m) from the speed of the rate-limiting step (i.e., k_{cat}), the difference between the

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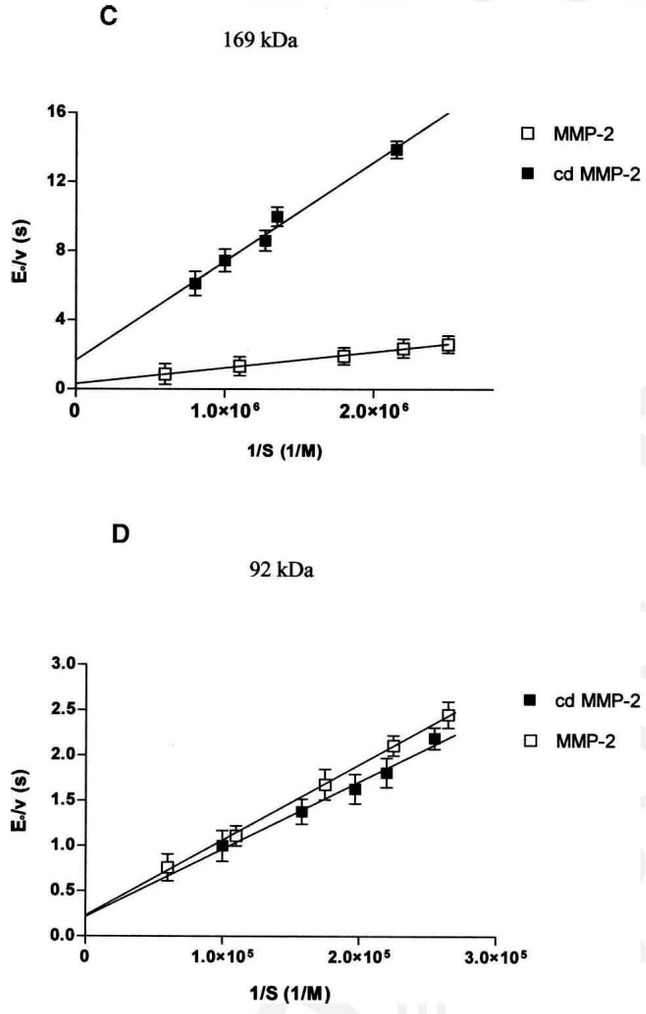

Figure 1. (*Continued*)

mechanisms by which whole MMP-2 processes the three species becomes even more drastic (see Table 1). Thus, MMP-2 shows a very high affinity for the recognition sites on the two putative α-1 and α-2 chains (as from the very low values of K_m; see Table 1), but an ~10-fold lower affinity for the 92-kDa form, leaving almost unchanged the speed of the rate-limiting step for the proteolytic cleavage (see Table 1). As a whole, these data suggest a much more dense structure for the α-1 and α-2 chains (with more specific and structured recognition sites), but a more disordered conformation for the 92-kDa species, which brings about the partial loosening of the recognition site with a decreased affinity for MMP-2 (see Table 1). It must be also stressed that our kinetic analysis has been limited to the first cleavage step of the three components of collagen IV, since this is the only process that follows the Michaelis–Menten approximation and that can be analyzed quantitatively. Fragments of lower molecular weight indeed are formed, but their kinetic building up cannot be followed appropriately with our detection methods.

Degradation of collagen type IV from human placenta by cdMMP-2

In the case of the catalytic domain of MMP-2, the lack of the hemopexin-like domain leads to a much lower value for k_{cat}/K_m, with respect to the whole enzyme for the two putative chains (by at least 1 order of magnitude), whereas no significant effect is observed for the catalytic efficiency toward the 92-kDa species (see Table 1). However, when we focus on values of k_{cat} and K_m some peculiar differences are observed for the various species with respect to the whole enzyme. Thus, for both chains the lower catalytic efficiency appears to be almost only due to a decreased k_{cat} (see Table 1). In the case of the 92-kDa species, the closely similar catalytic efficiency by cdMMP-2 with respect to the whole enzyme indeed reflects similar catalytic parameters (see Table 1).

Degradation of native collagen type IV from murine EHS sarcoma by whole MMP-2

The enzymatic processing of the native collagen type IV by whole MMP-2 is shown in Figure 2A at physiological F2

Table 1. *Catalytic parameters for the enzymatic processing at 37°C and pH 7.3 of collagen type IV from human placenta by the whole MMP-2 and by the catalytic domain of MMP-2*

	k_{cat}/K_m (M^{-1} sec^{-1})	k_{cat} (sec^{-1})	K_m (M)
207 kDa			
Whole MMP-2	1.4 (±0.2) × 10^6	1.8 (±0.3)	1.2 (±0.2) × 10^{-6}
Catalytic domain MMP-2	1.5 (±0.2) × 10^5	0.3 (±0.1)	2.0 (±0.3) × 10^{-6}
169 kDa			
Whole MMP-2	1.1 (±0.2) × 10^6	3.0 (±0.5)	2.7 (±0.4) × 10^{-6}
Catalytic domain MMP-2	1.7 (±0.3) × 10^5	0.6 (±0.2)	3.5 (±0.6) × 10^{-6}
92 kDa			
Whole MMP-2	1.2 (±0.2) × 10^5	4.6 (±0.7)	3.9 (±0.6) × 10^{-5}
Catalytic domain MMP-2	1.4 (±0.3) × 10^5	4.6 (±0.8)	3.4 (±0.7) × 10^{-5}

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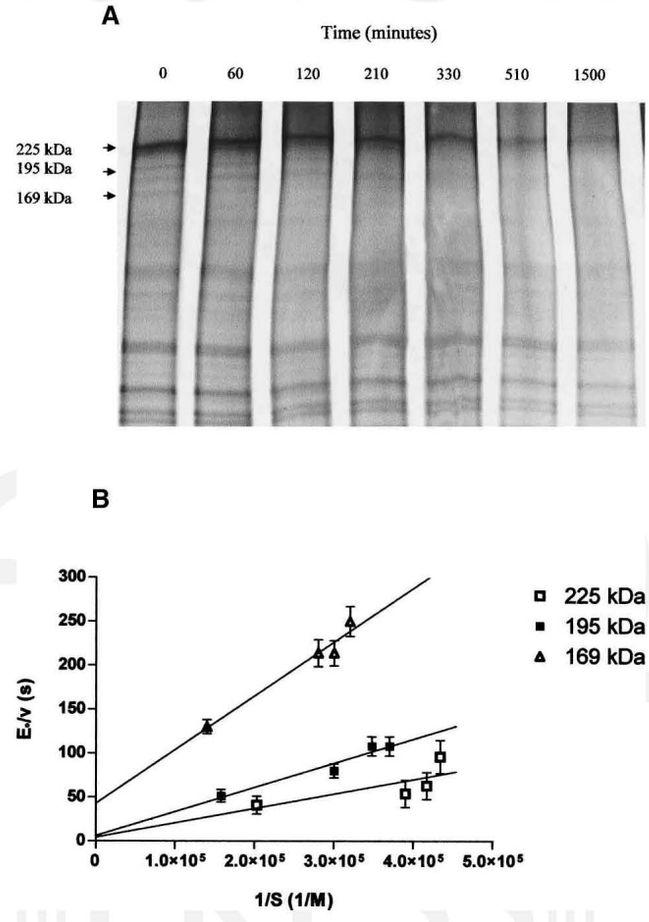

Figure 2. Enzymatic processing of native collagen IV by whole MMP-2 and double-reciprocal plots of different chains. (*A*) SDS-PAGE electrophoresis of the processing of native collagen type IV from murine Engelbreth-Holm-Swarm (EHS) sarcoma by whole MMP-2 as a function of time (0, 60, 120, 210, 330, 510, and 1500 min) at 37°C and pH 7.3. Fragments under investigation are indicated by their molecular mass: 225, 195, and 169 kDa. (*B*) Lineweaver–Burk plots of the processing by whole MMP-2 of different fragments of native collagen type IV from murine Engelbreth-Holm-Swarm (EHS) sarcoma, namely those characterized by 225 kDa (□), 195 kD (■), and 169 kDa (Δ) molecular mass. Catalytic parameters are reported in Table 2. (For further details, see text.)

temperature and pH as a function of the incubation time with the enzyme. The electrophoretic pattern shows a much larger number of species than in collagen type IV from human placenta (see Fig. 1A), probably due to the higher complexity of the native collagen type IV from the murine EHS sarcoma, as also reported by others (Mackay et al. 1990).

However, also in this case, only three species (namely, those corresponding to a 225-, 195-, and 169-kDa molecular mass, respectively) appear to be cleaved to a significant amount by whole MMP-2, while the other species with a lower molecular weight appear intact over the time range of the kinetic observation.

Also in the case of native collagen type IV, we observe for the first cleavage step a behavior compatible with the Michaelis–Menten mechanism (see Fig. 2B), and the catalytic parameters of the enzymatic processing are reported in Table 2. As is immediately obvious from data in Table 2, for all three species the catalytic cleavage efficiency by whole MMP-2 is drastically lower than that for species from collagen type IV from human placenta (see Fig. 1A; Table 1). In particular, for all three species the substantially reduced enzymatic activity seems mostly attributable to a dramatic decrease of k_{cat} values and only to a lesser extent to an increase for K_m, suggesting that the likely higher structural complexity of this type of collagen IV renders all the network much less susceptible to cleavage, even though the interaction of whole MMP-2 with native collagen type IV remains characterized by a relatively high affinity (see Table 2).

Increasing the temperature to 42°C brings about a partial unfolding of collagen IV (Dölz et al. 1988), and indeed we observe a marked enhancement of the enzymatic activity of MMP-2 (see Fig. 3). It is remarkable that

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Table 2. *Catalytic parameters for the enzymatic processing at 37°C and pH 7.3 of native collagen type IV (EHS) by whole MMP-2*

	k_{cat}/K_m (M^{-1} sec^{-1})	k_{cat} (sec^{-1})	K_m (M)
225 kDa MMP-2	5.0 (±0.4) × 10^3	0.1 (±0.07)	1.9 (±0.20) × 10^{-4}
195 kDa MMP-2	3.3 (±0.2) × 10^3	0.2 (±0.01)	5.0 (±0.4) × 10^{-5}
169 kDa MMP-2	1.7 (±0.1) × 10^3	0.02 (±0.001)	1.0 (±0.1) × 10^{-5}

the increase of k_{cat}/K_m (i.e., 1.2 × 10^4 $M^{-1}sec^{-1}$ at 42°C vs. 5.0 × 10^3 $M^{-1}sec^{-1}$ at 37°C; see Table 2) greatly exceeds what is expected on the basis of the activation enthalpy for MMP-2 (Fasciglione et al. 2000). It clearly suggests that an important contribution to this enhancement stems from the partial unfolding of the collagen IV network, supporting the idea that the lower catalytic efficiency toward native type IV collagen must be related to the tight assembly of the network.

Role of MMP-2 in the migration of neutrophils across a type IV collagen–coating membrane

F4 Figure 4 shows the extent of migration of neutrophils across a type IV collagen coating in the absence of any agent (column 1), in the presence of only LPS (as a chemoattractant factor) at a final concentration of 0.5 μg/mL (column 2), in the presence of Ilomastat (a wide range inhibitor of MMPs) at a final concentration of 100 μM (column 3), in the presence of both 0.5 μg/mL LPS and of active MMP-2 at different concentrations (columns 4–7), and in the presence of both 0.5 μg/mL LPS and the digestion products of 1 μM collagen IV by 40 nM MMP-2 (column 8). It is important to underline that neutrophil extracts do not contain appreciable amounts of MMP-2 activity, as from the gelatin zymographic analysis (see inset in Fig. 4), but only MMP-8 collagenase activity and MMP-9 gelatinase activity. As a matter of fact, data reported in columns 2 and 3 of Figure 4 indicate that (1) gelatinase activity is absolutely required for the neutrophil migration, as from the marked inhibitory effect exerted by Ilomastat (which is an inhibitor of MMPs; see Bendeck et al. 1996); and (2) Ilomastat, at concentrations used, does not inhibit MMPs production and it does not exert any toxic activity on neutrophils. The complete inhibition of MMPs produced during experiments in the presence of Ilomastat was confirmed by fluorimetric experiments performed using cell culture supernatant (data not shown). It should be underlined that Ilomastat inhibition is not irreversible, since during sample incubation with SDS the inhibitor is removed from MMPs that in turn become able to degrade gelatine during zymography (see in Fig. 4 the gelatine zymography corresponding to column 3). However, even more interesting is the effect observed if we add active MMP-2, since these data seem to support the idea that the presence of increasing amounts of active MMP-2 is depressing the migration of neutrophils across the type IV collagen–coated membrane (which is an oversimplified model for a basement membrane). The mechanism underlying such behavior can be explained by the production of lower molecular weight fragments of collagen IV, consequent to the cleavage

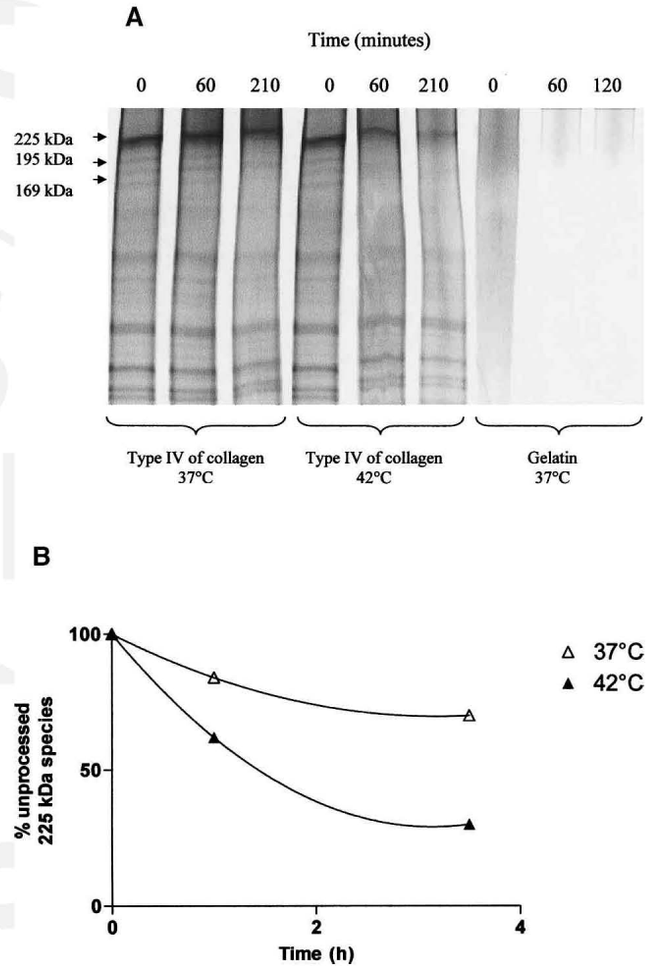

Figure 3. Effect of temperature on the enzymatic degradation of native type IV collagen by intact MMP-2. (*A*) SDS-PAGE electrophoresis of the processing of (1) native collagen type IV from murine Engelbreth-Holm-Swarm (EHS) sarcoma by whole MMP-2 after 0, 60, or 210 min, respectively, of enzyme incubation at 37°C (lanes *1–3*) and at 42°C (lanes *4–6*) at pH 7.3; and (2) gelatin after 0, 60 and 120 min of enzyme incubation at 37°C (lanes *7–9*). Fragments under investigation are indicated by their molecular mass: 225, 195, and 169 kDa. (*B*) Time dependence of the 225-kDa species degradation at 37°C (Δ) and 42°C (▲) by MMP-2. The ordinate refers to percentage of unprocessed species (%), while the evolution time is expressed in hours (h). The continuous lines are simply drawn through the points.

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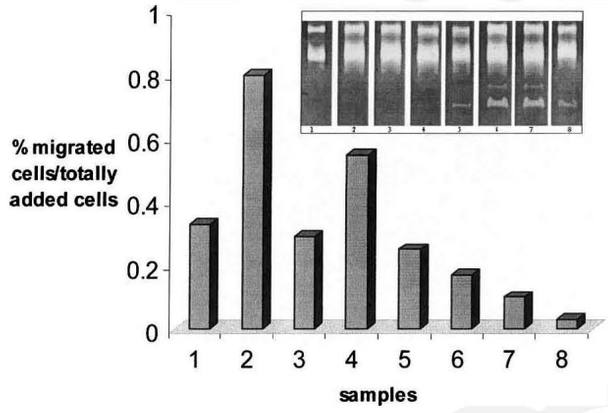

Figure 4. Extent of neutrophil migration across a barrier of collagen IV. Neutrophil chemotaxis through empty Transwell clots prepared with the indicated type IV collagen concentration was measured as described in Materials and Methods. The number of cells migrated into the lower chamber containing control buffer (1); in the presence of 0.5 μg/mL LPS (2); in the presence of 0.5 μg/mL LPS, 1 nM MMP-2, and 100 μM Ilomastat (3); in the presence of 0.5 μg/mL LPS and of active MMP-2 at different concentration (namely 1 nM in 4 and 3 nM in 5, 10 nM in 6, and 40 nM in c7); and in the presence of 0.5 μg/mL LPS and the product of collagen IV digestion by 5 nM MMP-2 (8) was determined using an LDH assay as described. The results are presented as a percentage of migrated cells vs. total cells. (*Inset*) Zymography of the neutrophil supernatant. Lanes refer to the analysis of the supernatant for the conditions reported in the corresponding bars of the main figure. Pro-MMP-9 (pMMP9), MMP-9 (MMP9), and MMP-2 (MMP2) are indicated on the *right* side of the inset. (For further details, see text.)

by MMP-2, which impairs the migration of neutrophils. This hypothesis finds support in data reported in column 8 of Figure 4, where the LPS-enhanced neutrophil migration (see Fig. 4, column 2) is dramatically inhibited by the addition of digestion products of collagen IV by MMP-2.

Similar results have been obtained in the case of membrane coated with native type IV collagen from EHS murine sarcoma, even though in this case all processes are much less evident because of the greater difficulty of neutrophils to migrate across this membrane coating (data not shown).

Role of the fibronectin-like domain of MMP-2 on the processing of collagen type IV from human placenta by whole MMP-2

F5 The SDS-PAGE electrophoretic pattern in Figure 5 shows the role of rCBD during the enzymatic processing of collagen type IV from human placenta by whole MMP-2. At the same incubation time it appears evident as the presence of rCBD (to a final concentration of 50 μM) mostly inhibits the proteolytic activity of MMP-2 on all three species of collagen type IV susceptible to cleavage. It clearly demonstrates that the affinity of rCBD is comparable to that of whole MMP-2, suggesting that the actual binding site for MMP-2 on all three species of collagen type IV from human placenta takes place through the fibronectin-like domain of MMP-2.

Discussion

It is well known that the macromolecular organization and the biomechanical stability of basement membrane is mainly determined by the network of type IV collagen (Kühn 1994); therefore, the mechanism of its proteolytic processing is of the utmost importance for a better comprehension of the tumor cells spreading across the basement membrane (Stetler-Stevenson et al. 1993) as well as of most remodeling and repair processes. Previous studies have shown that both collagen type IV from human placenta and native type IV collagen from EHS can be processed by MMP-2 (Eble et al. 1996), even though this process is sensibly temperature-dependent, being more efficient at 37°C than at lower temperatures (Mackay et al. 1990), probably because of some loosening on the tightness of the network and of the triple-helical assembly upon temperature rise (Dölz et al. 1988). Such a statement is further demonstrated in this article, where the catalytic efficiency (i.e., k_{cat}/K_m) is increased upon raising the temperature from 37°C to 42°C (see Fig. 3), well beyond what is expected on the basis of the activation enthalpy for MMP-2 activity (Fasciglione et al. 2000).

However, no attempt has been carried out up to now to correlate the integrity of collagen IV (and of other components of the basement membrane) with the capability of cells to migrate across the basement membrane for different physiological and pathological processes,

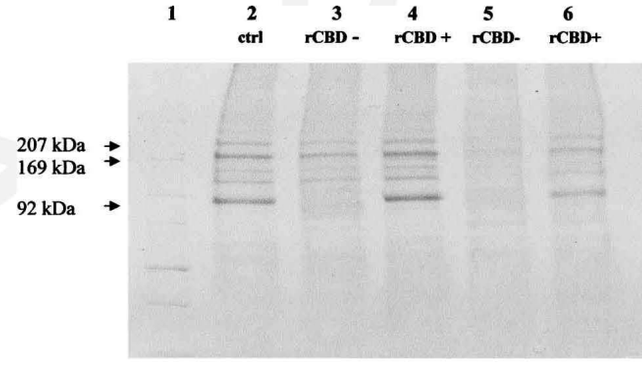

Figure 5. Effect of the presence of collagen binding domain of MMP-2 on the enzymatic processing of collagen IV from human placenta by whole MMP-2. SDS-PAGE electrophoresis of the processing of type IV collagen from human placenta by whole MMP-2 in the absence (lanes *3,5*) and in the presence of 50 μM rCBD (lanes *4,6*) at two different intervals after exposure to whole MMP-2, namely, 30 min (lanes *3,4*) and 45 min (lanes *5,6*). Lane *1* corresponds to molecular weight markers, and lane *2* is intact collagen type IV from human placenta. (For further details, see text.)

such as inflammatory response, angiogenesis, tumor dissemination, and others. This correlation can only be made by associating the detailed mechanism by which a MMP is cleaving and fragmenting different chains of collagen IV (and of other components), its molecular control and modulation by environmental conditions, and the effect of these events on the cellular migration.

In this article we present clear-cut evidence that gelatinase activity by MMP-9 indeed is important for the migration of neutrophils across a barrier represented by type IV collagen and that inhibition of its activity (upon addition of Ilomastat, a MMP inhibitor; see Bendeck et al. 1996) leads to a great depression of neutrophil migration (see column 3 of Fig. 4). However, this observation does not represent an absolute novelty, since previous papers suggested the importance of this role (Delclaux et al. 1996). On the other hand, a partially unexpected and very novel result is represented by the observation that MMP-2 plays a strong negative action for this process (see Fig. 4). Therefore, also on the basis of this functional cellular evidence, we have deemed it of the utmost importance to carry out a more quantitative kinetic analysis of this process by MMP-2 on collagen type IV from human placenta at 37°C and at pH 7.3, comparing it with the behavior of the catalytic domain of MMP-2, in order to characterize also the role of the hemopexin-like domain in the recognition and cleavage of collagen IV. The experiments have been carried out under nondenaturing conditions (since the temperature of 37°C is not denaturing collagen IV triple-helix, but only loosening it; see Dölz et al. 1988), even though the electrophoretic analysis is undertaken under denaturing conditions, so as to record the degradation of individual polypeptides. We focused our observations on three types of polypeptides, namely, those corresponding to a molecular mass of 207, 169, and 92 kDa in collagen type IV from human placenta; the first two are likely corresponding to the α-1 and α-2 chains, in view of the sequence mass determination (Hostikka and Tryggvason 1987; Soininen et al. 1987), whereas the third one is probably a peptide of the α-2 chain (P. van den Steen and G. Opdenakker, pers. comm.), resulting from the extraction treatment by pepsin with a possible unwound gelatine-like structure, as suggested by others (Kajimura et al. 2004).

The results obtained (see Fig. 1; Table 1) clearly indicate that the hemopexin-like domain does not play any essential role in the recognition process, whereas it turns out to be fairly important for determining the speed of the rate-limiting first cleavage event. This role is reminiscent of what has been reported for the processing of fibrillar collagen I by MMP-2 (Patterson et al. 2001), where also the role of the hemopexin-like domain seems more important for the cleavage process than for the substrate recognition. Therefore, data reported in Table 1 seem to indicate that a similar role is played by the hemopexin-like domain of MMP-2 in the processing of the triple-helical portion of collagen type IV from human placenta, as represented by the α-1 and α-2 chains (see above).

If we compare the catalytic parameters for the processing of the α-1 and α-2 chains (characterized by 207 kDa and 169 kDa, respectively) by whole MMP-2, a closely similar behavior is observed not only for the catalytic efficiency (as from k_{cat}/K_m; see Table 1) but also (within a factor of two) for the recognition mechanism and the rate-limiting cleavage event (i.e., K_m and k_{cat}; see Table 1), clearly suggesting a very similar proteolytic process by MMP-2 on the two chains of the triple-helical portion. However, an interesting result from Table 1 concerns the much-reduced catalytic efficiency of MMP-2 on the fragment of the α-2 chain, characterized by a molecular mass of 92 kDa, with a likely gelatine-like structural conformation. This feature seems mostly due to a more difficult recognition mechanism for both intact MMP-2 and its catalytic domain, as if the triple-helical arrangement (present in the other fragments, corresponding to the α-1 and α-2 chains) might favor the interaction between MMP-2 and the substrate. This difficulty is only partially compensated (only in the case of the catalytic domain; see Table 1) by a much faster rate-limiting step kinetic constant (possibly related to the much more unwound and unstructured conformation of the peptide), resulting in a similar value of k_{cat} for the intact MMP-2 and its catalytic domain (see Table 1).

As a whole, these results indicate that the hemopexin-like domain of MMP-2 is playing a very minor role in the substrate recognition process (as from the similar K_m values between intact MMP-2 and its catalytic domain; see Table 1). On the other hand, an effect linked to the presence or not of the hemopexin-like domain is observed for the k_{cat} of the two intact chains and it suggests that the hemopexin-like domain might play some role in the partial unwinding of the substrate when it is involved in the triple-helical arrangement. This statement seems supported by the evidence that no effect due to the lack of the hemopexin-like domain is observed for k_{cat} in the case of the 92-kDa fragment, which is likely not assembled in the triple helix and it is probably partially unwound (Kajimura et al. 2004).

We have also carried out an investigation of the proteolytic processing by whole MMP-2 of native collagen type IV from murine EHS sarcoma, which has been reported to be partially cleaved at 37°C by gelatinase A (Mackay et al. 1990; Eble et al. 1996). In this case, we have followed the enzymatic processing of fragments corresponding to 225 kDa, 195 kDa, and 169 kDa, respectively, which appear to be the only ones to be processed to a significant extent by MMP-2. It is very likely that these fragments refer to three types of chains, one of which (i.e.,

the 169-kDa fragment) has the same molecular mass as the α-2 chain, and the 195-kDa fragment has a molecular mass very close to that reported for the α-1 chain (Hostikka and Tryggvason 1987; Soininen et al. 1987). Catalytic parameters for the proteolytic cleavage of these three fragments by whole MMP-2 are reported in Table 2. It comes out very clear that the enzymatic efficiency is drastically lower than for the collagen type IV from human placenta (see Table 1) by ~3 orders of magnitude, especially if we compare the fragment that seems identical in the two types of collagen IV (i.e., the 169-kDa fragment). A closer comparison for the catalytic parameters of this fragment indicates that the low proteolytic efficiency (i.e., k_{cat}/K_m) is mostly due to a very slow rate constant for the cleavage rate-limiting step (i.e., k_{cat}), which is ~100-fold lower, and also in part to a 10-fold decrease for the substrate affinity (as from the higher K_m; see Tables 1, 2). The catalytic efficiency was even more reduced for the catalytic domain, impairing the possibility to obtain reliable catalytic parameters.

In order to have deeper information on the determinants of the recognition process, we have compared the fragmentation of collagen type IV from human placenta by whole MMP-2 in the presence and in the absence of the rCBD domain, which has been reported to simulate the interaction of the fibronectin-like domain of MMP-2 with both collagen I and collagen IV (Steffensen et al. 2002; Tam et al. 2004; Xu et al. 2005). The results clearly indicate a marked inhibition of the proteolytic processing (see Fig. 4) by rCBD, suggesting that indeed the whole MMP-2 interacts with the two chains of collagen type IV from human placenta through its fibronectin-like domain, as showed for collagen I (Tam et al. 2004).

As a whole, from these data it comes out that the complexity of the structural assembly of collagen type IV indeed represents a limitation for the proteolytic action of MMP-2 (and likely of other MMPs), and this conclusion seems strengthened by the effect of raising the temperature (see Fig. 3), which greatly exceeds the effect expected on the basis of the activation energy for MMP-2 activity (see Fasciglione et al. 2000), implying an important role deriving also from the partial unfolding of collagen IV (Dölz et al. 1988). This reduced enzymatic activity for the tight assembly of native collagen IV turns out to be referable to a reduced rate for the cleavage itself of the chains (which is dramatically slowed down in the native form from murine EHS sarcoma with respect to that from human placenta; see Tables 1, 2), whereas the recognition process (i.e., K_m) for the formation of the ES complex does not appear particularly compromised by the tight network formed by different chains. This feature, which turns out to be true only for the putative α-1 and α-2 chains of the type IV collagen network, suggests a possible direct role for MMP-2 in the modulation of the endoproteolytic process of the glomerular basement membrane responsible for the pathogenesis of the Alport's syndrome (Hudson et al. 2003). Thus, while from one side the action of MMP-2 on the chains of collagen IV allows the exposure of cryptic sites, which enhance the susceptibility of the enzymatic processing by other proteases (Xu et al. 2001), gelatinase A seems also responsible for a negative effect on neutrophil migration thanks to the production of anti-angiogenic fragmentation products (see Fig. 3). This observation clearly indicates the complexity of effects associated to the activity of MMP-2 (and probably of other MMPs), raising the question of whether a simple inhibitory effect on this activity can be advantageous for the control of pathological effects associated to it.

Materials and methods

Materials

Type IV collagen from human placenta (Sigma Chemical Co.) was dissolved in 0.1 M acetic acid at room temperature to a final concentration of 1 mg/mL. The suspension was centrifuged for 1 h at 10,000*g*, and the supernatant, containing the dissolved collagen, was used. The final amount of solubilized substrate has been quantified as described by Bradford (1976), employing soybean trypsin inhibitor as a reference.

Native type IV collagen from EHS (Sigma Chemical Co.) was dissolved in 0.1 M acetic acid at room temperature to a final concentration of 0.75 mg/mL. The suspension was centrifuged for 1 h at 10,000*g*, and the supernatant, containing the dissolved collagen, was used. The final amount of solubilized substrate has been quantified as described by Bradford (1976), employing soybean trypsin inhibitor as a reference.

MMP-2 recombinant proenzyme was either of commercial origin (R&D System) or a generous gift of Dr. Chris Overall (University of British Columbia); no significant functional difference has been detected between the two preparations after activation. The isolated purified MMP-2 was activated by incubating 0.1 mL of a 0.1 μg/mL progelatinase solution with *p*-aminophenyl mercuric acid (i.e., APMA) (Sigma Chemical Co) at 37°C for 30 min.

The catalytic domain (cdMMP-2) of human recombinant MMP-2 (Biomol International), containing the fibronectin-like domain, was dissolved in a solution of 50 mM Tris/HCl, 0.1 M NaCl, 10 mM $CaCl_2$ at pH 7.3.

The recombinant fibronectin-like domain (rCBD) of MMP-2 was a generous gift of Dr. Chris Overall (University of British Columbia) and its identity was verified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Xu et al. 2004) after running a small amount of rCBD on a small column of gelatin Sepharose (Sigma Chemical Co.) (Laemmli 1970).

The quenched fluorogenic substrate MCA-Pro-Leu-Gly-Leu-DPA-Ala-Arg-NH_2 was purchased from Calbiochem.

Lipopolysaccharide W *Escherichia coli* LPS was purchased from DIFCO Laboratories. Orthophenilendiamine (OPD) was purchased from Sigma Chemical Co. GM6001 (Ilomastat) was purchased from BioMol.

Activity assay

The active amount of the enzyme (MMP-2) was determined by gelatin zymography and by the fluorimetric assay, as described

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by others (Knight et al. 1992), following the progressive decrease of hydrolysis of the quenched fluorogenic substrate MCA-Pro-Leu-Gly-Leu-DPA-Ala-Arg-NH_2 upon addition of Batimastat (BB-94), a peptidomimetic inhibitor (kindly provided by British Biotech Pharmaceutical), which stoichiometrically inhibits MMPs.

Kinetics of digestion of collagen type IV from human placenta and of native collagen type from murine EHS sarcoma by whole MMP-2 and cdMMP-2

For substrate fragmentation kinetics, activated whole MMP-2 was added to collagen type IV solutions from human placenta or from murine EHS sarcoma, at a final concentration of 10 pM, while the catalytic domain of MMP-2 was added to collagen solutions at a final concentration of 15–20 pM. The kinetics was carried out in 50 mM Tris/HCl, 0.1 M NaCl, 10 mM $CaCl_2$ at pH 7.3, employing different concentrations of collagen type IV (spanning between 1 μM and 4 μM). Kinetics was carried out keeping the mixtures at 37°C (or at 42°C) and harvesting small aliquots at different time intervals. Reactions were stopped by the addition of SDS-PAGE loading buffer containing 20 mM EDTA and frozen to −80°C until used.

The aliquots in reducing sample buffer were separated on 4%–15% gradient SDS-PAGE gels, which were stained using 0.5% Coomassie Blue and destained in 10% acetic acid and 40% methanol until substrate bands were clearly visible or employing a Silver Staining solution. The broad spectrum protein markers (Bio Rad) were used as molecular weight standards.

Human neutrophils isolation

Neutrophils were isolated from healthy donors volunteers. Whole blood was diluted (blood–PBS1× ratio 1:4) and stratified on Ficoll separating solution, then centrifugated at 1400 rpm for 30 min at 4°C. Plasma and mononuclear cells were then removed by aspiration, and the red blood cells were lysed by adding 18 mL of ice-cold water for 30 sec; thereafter 2 mL of PBS 10× were added and suspension was centrifuged (1600 rpm for 5 min at 4°C) in order to remove RBC membrane debris and hemoglobin.

The neutrophil-containing pellet was then washed twice at 1600 rpm for 5 min at 4°C and resuspended in RPMI 1640 (EuroClone) medium supplemented with BSA (0.2%) to a final concentration of 4×10^6 cells/mL.

Viability of purified cells was measured by Trypan blue dye exclusion count, and it was found always >95%.

Chemotaxis assay

Human neutrophil chemotaxis assay was measured in Falcon 24-well plates containing Transwell inserts with 3.0 μm pore diameter (Roberto Da Costa et al. 2003). Transwell inserts were coated overnight with type IV collagen from human placenta (Sigma Chemical Co.) or from murine EHS sarcoma previously dissolved in 0.1 M acetic acid at room temperature and diluted with 1× PBS to a final concentration of 10 μg/mL.

Lower chambers contained 500 mL of RPMI 1640 medium supplemented with 0.5% BSA, to which we have added (for different experiments):

- LPS to a final concentration of 500 ng/mL as a positive control;
- LPS 500 ng/mL and exogenous MMP-2 at different concentrations spanning between 1 and 40 nM;
- LPS 500 ng/mL, exogenous MMP-2 to a final concentration of 1 nM and Ilomastat (a powerful inhibitor of MMPs, see Bendeck et al. 1996) to a final concentration of 100 μM;
- LPS 500 ng/mL and the digestion product (250 μl) of collagen IV by 5 nM MMP-2. In this experiment, type IV collagen (20 μg/mL) was digested for 6 h with MMP-2 to obtain 50% degradation measured in SDS-PAGE; after degradation, MMP-2 was stoichiometrically inhibited with Ilomastat, a synthetic MMPs inhibitor, as confirmed by fluorimetric assay. This experiment was performed on uncoated Transwell in order to directly measure the effect of type IV collagen digested fragments on MMP-independent LPS-mediated chemiotaxis.

In the upper compartment 250 μL of neutrophils (10^6 cells/well) were added.

Cells were allowed to transmigrate for 4 h at 37°C in a humidified 5% CO_2 incubator (Hanson and Quinn 2002).

To evaluate the transmigrated neutrophils, the Transwell inserts were removed after washing the lower part of membrane and the bottom solution was collected and centrifuged; cell pellets were then suspended in a solution containing Triton X-100 to a final concentration of 0.5%, OPD 600 μg/mL, and H_2O_2 6 μl/mL in 1× PBS.

Diluted concentrations of neutrophils, from 10^6 cells/well to 6×10^4 cells/well were used to obtain a standard curve.

The oxidation of OPD performed by myeloperoxidase contained in neutrophils was stopped by adding 40 μL of 2 M HCl; the reading was performed at 492 nm in a spectrophotomer (Jasco V-530). Any experiment was performed in triplicate at least with two different blood samples from different donors.

Role of the fibronectin-like domain of MMP-2 (rCBD) on the processing of collagen type IV from human placenta by whole MMP-2

The fibronectin-like rCBD domain of MMP-2 (at a final concentration of 50 μM) was incubated with collagen type IV from human placenta (1 mg/mL) for 30 min at 37°C to allow the interaction to occur. After this time period, MMP-2 was added to the mixture and the solution was put for another 30 min at 37°C. At the same time, three additional control solutions were prepared, namely, (1) collagen type IV alone, (2) collagen type IV incubated for the same time with MMP-2 at 37°C, and (3) a solution of collagen type IV with 50 μM rCBD but without MMP-2.

Reactions were stopped by the addition of SDS-PAGE loading buffer containing 20 mM EDTA and frozen to −80°C until used. The different samples were visualized by SDS-PAGE electrophoresis.

Kinetic analysis

Electrophoretic spots, corresponding to different aliquots at different time intervals, were analyzed by a laser densitometer (LKB 2202 UltraScan) and their intensity was calibrated (in order to obtain concentration values) using standard substrate solutions. For the different species, the substrate disappearance rates were derived at each concentration of type IV collagen employed.

The measurement of the initial velocity was referred to a time period of 1 h for collagen type IV from human placenta and 3 h from native collagen type IV over which <10% of the substrate

was degraded during the assay, and in any case the analysis was limited to the time interval over which linearity of the rate was observed. It ensured a steady-state condition for the first cleavage step, and it was a prerequisite for the subsequent analysis step. The verification for the applicability of the Michaelis–Menten approximation to the first cleavage step and the determination of the catalytic parameters were undertaken by a global analysis of experimental data according to both the Lineweaver–Burk equation

$$\frac{E_0}{v} = \frac{K_m}{k_{cat}} \cdot \frac{1}{[S]} + \frac{1}{k_{cat}} \quad (1)$$

and the Eadie–Hofstee equation

$$\frac{v}{E_0} = k_{cat} - \frac{v}{E_0 \cdot [S]} \cdot K_m \quad (2)$$

where E_0 is the total enzyme concentration, v is the actual rate (expressed as **M**/s), K_m is the Michaelis–Menten equilibrium constant (expressed as **M**), k_{cat} is the rate-limiting step kinetic constant (expressed as sec ^{1}), and $[S]$ is the substrate concentration.

Acknowledgments

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Characterization of the Mechanisms by which Gelatinase A, Neutrophil Collagenase, and Membrane-Type Metalloproteinase MMP-14 Recognize Collagen I and Enzymatically Process the Two α-Chains

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The turnover of native collagen has been ascribed to different members of the matrix metalloproteinase (MMP) family. Here, the mechanisms by which neutrophil collagenase (MMP-8), gelatinase A (MMP-2), and the ectodomain of MT1-MMP (ectMMP-14) degrade fibrillar collagen were examined. In particular, the hydrolysis of type I collagen at 37 °C was investigated to identify functional differences in the processing of the two α–chain types of fibrillar collagen. Thermodynamic and kinetic parameters were used for a quantitative comparison of the binding, unwinding, and hydrolysis of triple helical collagen. We demonstrate that the MMP family has developed at least two distinct mechanisms for collagen unwinding and cleavage. MMP-8 and ectMMP-14 display a similar mechanism (although with different catalytic parameters), which is characterized by binding (likely through the hemopexin-like domain) and cleavage of α–1 and/or α–2 chains without distinguishing between them and keeping the gross conformation of the triple helix (at least during the first cleavage step). On the other hand, MMP-2 binds preferentially the α–1 chains (likely through the fibronectin-like domain, which is not present in MMP-8 and ectMMP-14), grossly altering the whole triple helical arrangement of the collagen molecule and cleaving preferentially the α–2 chain. These distinctive mechanisms underly a drastically different mode of interaction with triple helical fibrillar collagen I, according to which the MMP domain is involved in binding. These findings can be related to the different role exerted by these MMPs on collagen homeostasis in the extracellular matrix.

Keywords: collagen I; cleavage; binding; circular dichroism; kinetics

Introduction

The collagen family represents a group of different extracellular matrix proteins, which provide strength and rigidity to the connective tissue due to the presence of a particular quaternary structure (called the triple helix structure).[1] Fibrillar collagens have the capacity to self-associate, generating a supramolecular organization of 67 nm D periodic fibrils.[2,3] In particular, its monomer consists of three left-hand α-chains that are coiled around each other into a right-hand triple helix. This characteristic folding requires a repeated sequence, that is Gly-*X*-*Y*, where *X* and *Y* positions are mainly proline and 4-trans-L-hydroxyproline residues, respectively.[4] This assembly is particularly observed in fibrillar type I collagen, which is one of the main components of the extracellular matrix.[1,3]

Abbreviations used: MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; CBD, collagen binding domain; ectMMP-14, ectodomain of MMP-14.

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Collagen matrix degradation and remodeling are important aspects of normal tissue homeostasis, because the alteration of collagen turnover is often associated with the progression of diseases like fibrosis, arthritis, cancer, atherosclerosis, and aneurysm.[5–8] Moreover, the degradation of extracellular collagen drives cell migration, proliferation, and differentiation through the uncovering of collagen cryptic molecular determinants.[9–11]

The complex triple helical fold renders collagen extremely resistant to proteolytic attack and only a few classes of proteases are able to degrade this molecule; among them, the most prominent members belong to the matrix metalloproteinases (MMPs) family.

The matrix metalloproteinases are a family of Ca^{2+} and Zn^{2+}-dependent neutral endopeptidases collectively capable of degrading essentially all components of the extracellular matrix. The MMPs, which are synthesized by connective tissue cells, are important for the remodeling of the extracellular matrix that accompanies physiologic processes (such as uterine involution, bone resorption, and wound healing) as well as pathologic processes (such as tumor growth and diffusion).[12] This wide spectrum of substrates and activities is reflected in the complexity of their recognition and degradation mechanisms.

MMPs are multidomain enzymes and they can be further subdivided (according to their domain composition and their efficiency in degrading substrates) into five groups, namely: (a) matrilysins, (b) collagenases (e.g., MMP-8), (c) gelatinases (e.g., MMP-2), (d) stromelysins, and (e) membrane-type MMPs (e.g., MMP-14).[13]

The general structure of MMPs includes a signal peptide, a propeptide domain, a catalytic domain with a highly conserved zinc-binding active site, and a hemopexin-like domain. In particular, collagenases, as well as the soluble form of members of membrane-type (MT)-MMP group, display a catalytic domain linked to the hemopexin-like domain via a short hinge peptide, whereas gelatinases (namely MMP-2 and MMP-9) also contain fibronectin type II inserts (consisting of three tandem copies of the fibronectin type II-like module) within a loop in the catalytic domain. Further, whole MT-MMPs contain at the C-terminal end of the hemopexin-like domain a transmembrane domain, which can be cleaved off under physiological conditions, bringing about the shedding of soluble forms in the extracellular matrix.[13,14] In the past few years it has been reported that, beside the catalytic site, regions far from the active site (called exosites) play an important role in facilitating both substrate binding and proteolysis.[15]

Within the MMP family only three subclasses (namely collagenases, gelatinases, and MT-MMPs) are involved in native fibrillar collagen degradation.[16–19] In view of their importance, these enzymes have been deeply studied in the past 20 years. In particular, most of the information on their enzymatic mechanism was elucidated by studies on triple helix peptides, illustrating that gelatinases possess distinct features with respect to collagenases and MT-MMPs.[20–24]

On the other hand, only few data are available on the MMP mechanisms of cleaving native collagens; among them, we must mention the comparison of collagenolytic activity at 25 °C between MMP-2 and MMP-14,[23] the study of the unwinding properties of MMP-1,[25] and the enzymatic mechanism of MMP-8 on fibrillar collagen at 37 °C.[26,27] Nevertheless, accurate mechanisms for binding, distorting, and hydrolyzing triple helical structures by these three class of MMPs are still far from being understood, mainly because of the lack of structural information on fibrillar collagen. However, very recently some structural information on fibrillar collagen has become available,[3] confirming that the substrate-binding site of MMPs is too narrow to accommodate triple helical collagen, as already postulated on the basis of the three-dimensional structural data of several MMPs.[15,25,28–30] As a consequence, it has been recently supposed that MMP exosites may induce at least a local unwinding of the triple helix to allow every collagenolytic step to take place.[21,31,32]

Several factors contribute to rendering the elucidation of the mechanism of collagen processing by MMPs a very challenging task. First of all, we must mention that collagen structure is temperature dependent (with a melting point close to the physiological temperature of 37 °C) and the low thermostability of collagen was suggested to be important for the independent mobility of individual helices required for various biological functions.[33] NMR studies have shown that at 37 °C triple helices have backbone motilities even in fibrils,[34] and it has been established that the energetically preferred conformation of type I collagen is more toward a random coil rather than toward a compact triple helix.[35] A second point is represented by the fact that little information is available on the rearrangements that occur upon the MMP-collagen interaction to catalyze each hydrolysis of the three cleavable peptide bonds, leading to the formation of the 1/4 and 3/4 fragments.[23,25,36]

To contribute to some of the points above, we compared at 37 °C the hydrolysis of native collagen I by neutrophil collagenase MMP-8, gelatinase A MMP-2, and ectMMP-14, aiming to describe quantitatively the different mechanisms by which these MMPs interact with and enzymatically process the two types of α–chains of fibrillar type I collagen.

The present study allows the assertion that the MMP family has evolved at least two different mechanism in processing collagen type I: MMP-2 proteolytic activity requires a substantial unwinding of the collagen I molecule, whereas the cleavage process by MMP-8 and ectMMP-14 can occur on an almost intact triple helix. In addition, the preferential enzymatic action on the α-2 chain by MMP-2, which is not observed in the case of MMP-8 and ectMMP-14, suggests that as long as the triple helical structural arrangement is maintained, no preferential enzymatic action is exerted on either of the two

types of chains. This information should represent an important step toward the elucidation of the molecular determinants controlling collagen catabolism and they could be used to address new therapeutic strategies to modulate collagen destruction in degenerative diseases.

Results

We have previously shown a marked temperature dependence for the intrinsic enzymatic activity of MMP-2, MMP-9, and MMP-8[37] and for the proteolytic activity of MMP-8 on natural substrate.[27] Because most of the biochemical studies on the enzymatic activity of MMPs have been performed at 25 °C[21,23–25,32] and the biological relevance of collagen proteolysis at 37 °C has been reported in several cell culture experiments,[18,38] we decided to carry out an investigation on collagen I degradation by MMP-2, MMP-8, and ectMMP-14 at 37 °C, under conditions as close as possible to the physiological conditions. Obviously, at 37 °C the structure of the collagen triple helix is different from that observed at 25 °C, as previously reported on the basis of CD ellipticity degree at 223 nm.[27] As a consequence, after 3 h of incubation of collagen with trypsin at 37 °C a moderate enzymatic processing can be observed, which starts only after 2 h (Figure 1, lane 3). However, this result does not imply that at 37 °C the structure is less "native" than at lower temperatures, as long as whatever happens to the triple helical assembly is fully reversible. Therefore, to validate this occurrence, we incubated collagen I at 37 °C for 24 h and then brought the sample back to 28 °C (the highest temperature reported in the literature). Subsequent exposure to trypsin did not show any evidence of enzymatic processing (Figure 1, lane 2), thus demonstrating that at 37 °C the conformation is somewhat looser, as expected, but still native, as indicated by the full reversibility with temperature of the compactness of the triple helix. Importantly, under these conditions, in the presence of collagen all three MMPs are perfectly stable without undergoing any autocatalytic activity over 60 min at 37 °C (data not shown), which is the period needed to obtain kinetic data for the calculation of catalytic parameters.

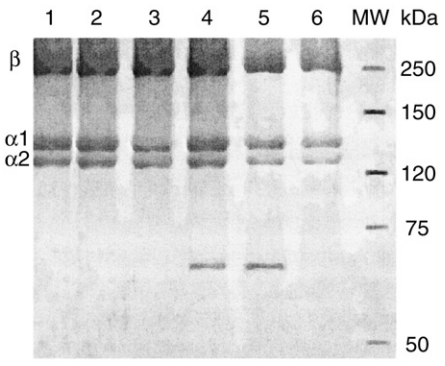

Figure 1. SDS-(7.5%) PAGE electrophoresis of collagen I degradations. In the absence of any enzyme (lane 1), by trypsin (10 nM) for 3 h at 28 °C after 24 h incubation at 37 °C (lane 2), by trypsin (10 nM) for 3 h at 37 °C (lane 3), by MMP-2 (2 μM) fully inhibited by Batimastat for 3 h (lane 4), by trypsin (10 nM) for 3 h in the presence of MMP-2 (2 μM) fully inhibited by Batimastat (lane 5), and by active MMP-2 (10 pM) for 3 h at 37 °C (lane 6). Lane 7 reports MW markers. For further details, see the text.

The catalytic action of MMP-2, MMP-8, and ectMMP-14 on α-1 and α-2 chains

The degradation of collagen occurs through an ordinate series of cleavage events[26] and the first step (corresponding to the formation of 3/4 and 1/4 fragments)[36] is crucial because of the fairly slow rate of collagen turnover within the cartilage.[39] We obtained catalytic parameters on each α-chain (i.e., k_{cat}/K_m, k_{cat}, and K_m) for the three MMPs by following the time dependence of substrate disappearance as a function of substrate concentration under nondenaturing conditions to sort out the action of MMPs on the two different chains of collagen I.

Figure 2 shows an example of the electrophoretic pattern of collagen I degradation observed for each MMP investigated. In the gels we can see three bands (identified as β, α-1, and α-2), which change intensity as a function of time of incubation with MMP-2, MMP-8, and ectMMP-14 at 37 °C. Although these three species have never been deeply characterized, there is a general agreement that α-1 and α-2 forms are the two types of chains, forming the actual triple helical collagen I molecule (in a 2:1 ratio), while the β form is a cross-linked polymer of fibrils formed by two triple helical collagen I molecules linked through intermolecular telopeptide interactions and interlysyl bonds.[1,40]

We followed the time evolution for the first hour of the band intensity for all three species after incubation with each MMP at 37 °C, determining the enzymatic processing only for the two chains (and not for the β form) at different concentrations of collagen I. The apparent cleavage rate was measured at each employed concentration of collagen and it was normalized to the amount of active enzyme. As expected, in all cases the resulting reciprocal of the steady-state velocity as a function of the reciprocal of α-1 and α-2 concentration is linear (Figure 3) and this evidence indicates that, at least for the first step, the enzymatic cleavage of α-1 and α-2 chains of collagen I follows the Michaelis-Menten mechanism. The derived parameters k_{cat}, K_m, and k_{cat}/K_m are reported in Table 1.

Looking at the parameters for both α chains reported in Table 1, it appears clear that MMP-2, which is characterized by weak collagenase activity at 25 °C,[15] shows powerful collagenase activity at the physiological temperature of 37 °C, even higher than the collagenase itself (i.e., MMP-8). However, this observation is not unexpected in view of the reported very high temperature dependence (corresponding

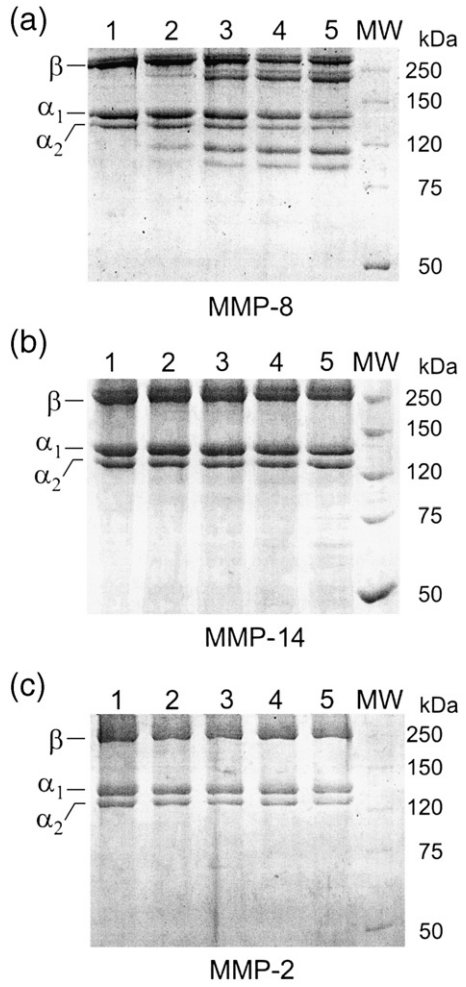

Figure 2. SDS-(7.5%) PAGE (7.5%) electrophoresis of the degradation kinetics of bovine collagen I at 37 °C, pH 7.3, by MMP-8 (a), ectMMP-14 (b), and MMP-2 (c). Different collagen chains are indicated; the incubation time is: (for a and b) 0 min (lane 1), 20 min (lane 2), 40 min (lane 3), 50 min (lane 4), 60 min (lane 5); (for c): 0 min (lane 1), 10 min (lane 2), 20 min (lane 3), 30 min (lane 4), 40 min (lane 5). The last column reports MW markers. For further details, see the text.

to a high Arrhenius activation energy) for the catalytic behavior of MMP-2 compared to other MMPs.[37]

At 37 °C MMP-8 and ectMMP-14 show similar behavior for the two different chains both for k_{cat} and for K_m, suggesting that they do not distinguish between the two types of chains. However, some difference between MMP-8 and ectMMP-14 can be observed for the catalytic parameters, because MMP-8 shows a somewhat higher affinity for substrates, whereas ectMMP-14 appears somewhat faster for the cleavage rate-limiting step (see Table 1).

A drastic difference can be noticed for the action of MMP-2 on the different chains of collagen I at 37 °C. Thus, beside a much higher overall catalytic efficiency of MMP-2 with respect to MMP-8 and ectMMP-14, the value of k_{cat}/K_m is somewhat different for the two chains, being a little higher toward the α-2 chain than the α-1 chain (see Table 1). However, it must be stressed that this feature is connected to a different contribution of k_{cat} and K_m for the different substrates. Thus, the slower k_{cat}/K_m for the α-1 chain of collagen I is fully attributable to the very slow k_{cat} (partially compensated by a very low value for K_m, referrable to a strong interaction of MMP-2 with the recognition site(s) on the α-1 chain), while in the case of the α-2 chain a very fast k_{cat} is observed (see Table 1) with a fairly high K_m value. This feature clearly indicates a completely different modulation mechanism for substrate cleavage with respect to the partner chain α-1, envisaging a different role of the two chains in the interaction with MMP-2. As a whole, this result indicates that

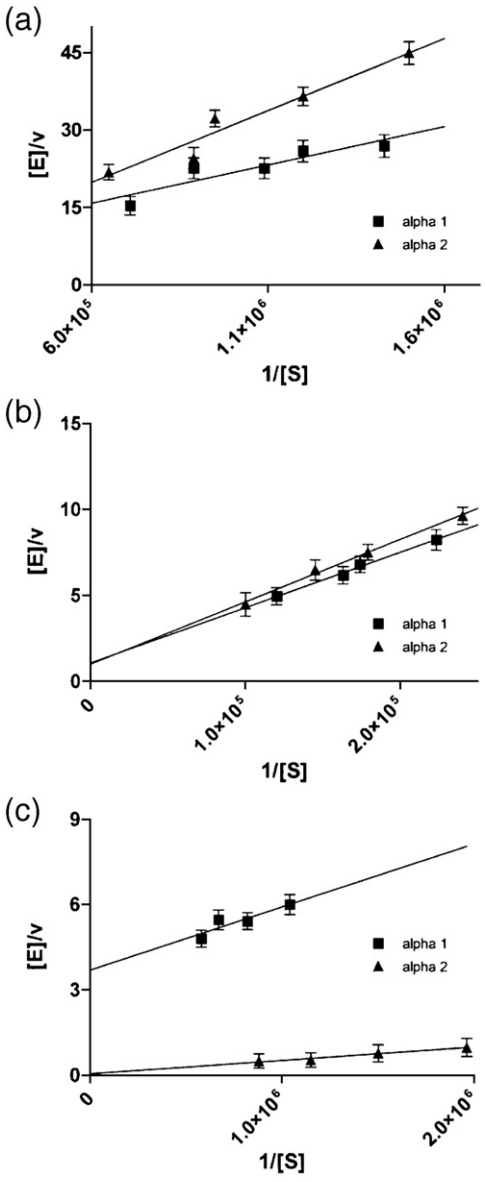

Figure 3. Lineweaver-Burk double-reciprocal plots of the steady-state velocity versus substrate concentration for the first-step cleavage of bovine collagen I by MMP-8 (a), ectMMP-14 (b), and MMP-2 (c). Solid lines are least-squares fitting of data according to equation (1). For further details, see the text.

Table 1. Catalytic parameters for the enzymatic cleavage of α-1 and α-2 chains of collagen I at 37 °C by different MMPs

Enzyme	Collagen chain	k_{cat}/K_m ($M^{-1}s^{-1}$)	k_{cat} (s^{-1})	K_m (M)
MMP-8	α-1	$5.0(\pm0.6)\times10^4$	0.32 ± 0.05	$6.4(\pm0.7)\times10^{-6}$
	α-2	$3.3(\pm0.4)\times10^4$	0.31 ± 0.05	$9.4(\pm1.1)\times10^{-6}$
ectMMP-14	α-1	$3.1(\pm0.4)\times10^4$	1.05 ± 0.12	$3.4(\pm0.4)\times10^{-5}$
	α-2	$2.8(\pm0.3)\times10^4$	0.99 ± 0.11	$3.5(\pm0.4)\times10^{-5}$
MMP-2	α-1	$4.5(\pm0.5)\times10^5$	0.27 ± 0.03	$6.0(\pm0.8)\times10^{-7}$
	α-2	$2.0(\pm0.3)\times10^6$	70.1 ± 8.1	$3.5(\pm0.4)\times10^{-5}$

MMP-2 preferentially interacts with the α-1 chain, whereas the α-2 chain is more susceptible to cleaveage.

Unwinding activity

Kinetics of collagen degradation was performed on the native triple helix and the analysis by SDS–PAGE electrophoresis allows information to be obtained on each chain separately. Therefore, to combine the enzymatic action on the individual chains to the overall effect on the triple helix during the cleavage process we recorded the CD spectra of type I collagen during proteolysis by one of the three MMPs investigated.

MMP-8 and ectMMP-14

In the case of MMP-8 very minor changes can be detected for the CD spectra over the time period during which the first cleavage step into the 1/4 and 3/4 fragments takes place,[26] suggesting that the unwinding of the molecule, if any, is very limited. A similar behavior is also shown by ectMMP-14 (Figure 4(a)): in fact, at the beginning of the collagen I degradation process, no meaningful change was observed. Thus, also in this case, the unwinding of collagen by ectMMP-14 action during the first step eventually involves only a limited portion of the triple helix without perturbing the overall structure. Further, agarose-entrapped collagen I incubated with fully inhibited ectMMP-14 and MMP-8 did not show any variation of the CD spectrum (data not shown), clearly indicating that binding itself does not perturb significantly the triple helical structure.

An analogous behavior was previously suggested for collagenase MMP-1, proposing that the enzyme locally unwinds triple helical collagen before hydrolyzing the peptide bonds.[25] On the other hand, after the first cleavage step further degradation of collagen I occurs by MMP-8 and ectMMP-14, accompanied by a large variation of the CD spectrum with a great decrease in peak intensity at 223–225 nm (see Figure 4(a)).[26]

Gelatinase MMP-2

Unlike MMP-8 and ectMMP-14, the exposure of the agarose gel-entrapped collagen I to MMP-2 brings about large CD spectral changes from the beginning with a rapid decrease of ellipticity (see Figure 4(b)). These results agree with that reported by others[23] after the addition of the collagen binding domain (CBD) of MMP-2 to collagen I (which indeed interacts with collagen I without cleaving it),[23] and they are grossly different from that shown by the other two MMPs, where the initial cleavage step is not accompanied by any relevant rearrangement on collagen structure (see Figure 4(a)).[26]

Spectra have been recorded in Figure 4(a) and (b) under different experimental settings. Thus, because the process reported in Figure 4(b) occurs over a much shorter time range than that reported in Figure 4(a), in the first case it was not possible to accumulate the same number of spectra as for Figure 4(a), resulting in an apparent different noise degree.

Therefore, the difference observed for the time evolution of CD spectra can be explained by assuming that MMP-2 unwinds the triple helix from the beginning of the substrate degradation process. This variation should be associated with the preferential interaction of MMP-2 with collagen I through the fibronectin-like domain (corresponding to the CBD), absent in MMP-8 and in ectMMP-14

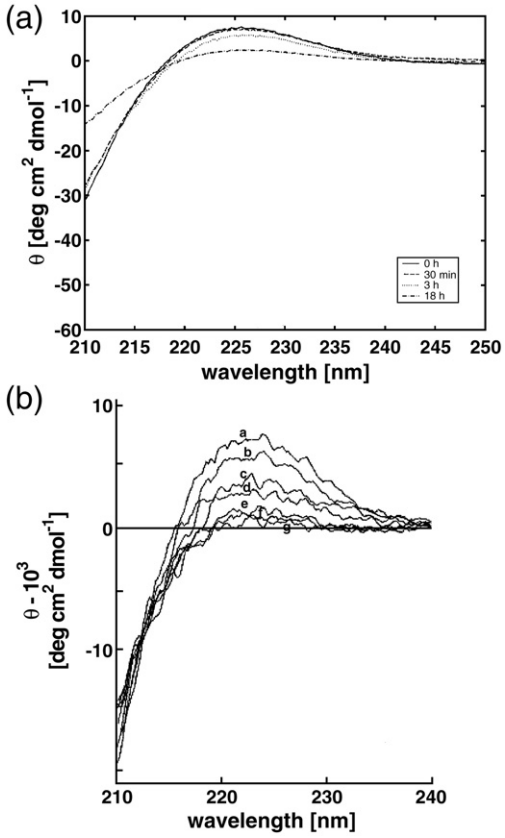

Figure 4. Circular dichroism spectra of agarose gel-entrapped collagen I at different time intervals after exposure to 10 pM ectMMP-14 (a) and 10 pM MMP-2 (b) at 37 °C, pH 7.3. In (a) time intervals are indicated, while in (b) time intervals are as follows: (a) 0 min, (b) 30 min, (c) 60 min, (d) 120 min, (e) 240 min, (f) 360 min, (g) 480 min. It must be remarked that spectra reported in (b) are the averages of only two spectra because of the kinetics. For further details, see the text.

(which instead should preferentially interact with collagen I through the hemopexin-like domain) and that only the interaction with the fibronectin-like domain induces a gross alteration of the triple helical structural arrangement, which is then followed by substrate cleavage and degradation (Figure 4(b)).

Is cleavage needed to induce the triple helix unwinding of collagen I by MMP-2?

To understand the MMP-2 collagen interaction itself as well as the subsequent effect on triple helix unwinding, we performed CD experiments employing fully inhibited MMP-2.

Batimastat was employed as inhibitor and the full inhibition of MMP-2 was validated with a synthetic substrate, but it was further reinforced by the observation (reported in Figure 1, lane 4) that after 3 h of incubation at 37 °C with inhibited 2 μM MMP-2 no degradation of collagen I was detected. Over the same period at the same temperature, 10 pM active MMP-2 brings about an extensive degradation of collagen I (see Figure 1, lane 6).

As shown in Figure 5, a variation of the CD spectra of gel-entrapped collagen I is detected even in the presence of fully inhibited MMP-2, indicating that the triple helix unwinding activity is present even in the absence of proteolytic cleavage. We carried out the experiment both in the presence of a substoichiometric amount of inhibited MMP-2 (1:10 ratio between MMP-2 and collagen I) and in the presence of a 2-fold excess of inhibited MMP-2 over collagen. The extent of triple helix unwinding (as indicated by the disappearance of the ellipticity at 223 nm, see Figure 5) indeed is dependent on MMP-2 concentration, because in the presence of substoichiometric amounts of MMP-2 only a partial unwinding can be observed (see spectrum b in Figure 5), while an excess of MMP-2, which guarantees binding saturation of the high-affinity site on collagen I (see K_m for the α-1 chain in Table 1),[41]

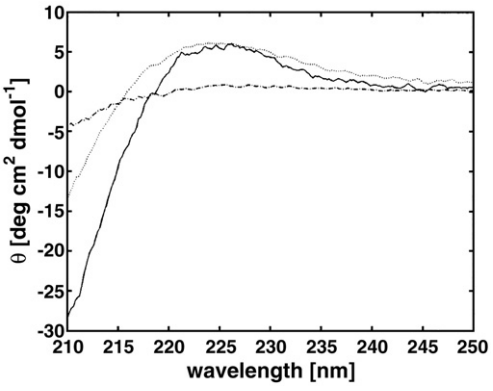

Figure 5. Circular dichroism spectra of agarose gel-entrapped collagen I at a concentration of 1 μM at 37 °C (dotted spectrum), after the addition of 0.2 μM MMP-2 fully inhibited by Batimastat (solid spectrum), and after the addition of 2 μM MMP-2 fully inhibited by Batimastat (dashed spectrum). For further details, see the text.

brings about an almost complete unwinding of the triple helix (see spectrum c in Figure 5). These ellipticity changes occur immediately after the addition of inhibited MMP-2 and no further spectral change is observed even after 2 h from the addition, ensuring that (i) binding equilibrium is attained and (ii) no proteolysis of collagen is taking place. The observation that fully inhibited MMP-2 induces a dramatic decrease of ellipticity immediately after addition, whereas 10 pM active MMP-2 requires several hours for an analogous effect, indicates that: (i) the effect reported in Figure 5 (where the enzyme/substrate ratio is 1:10 for spectrum b and 2:1 for spectrum c) is due to the binding (without substrate cleavage) of one molecule of MMP-2 for each molecule of triple helical collagen I; and (ii) the phenomenon observed in Figure 4(b) (where the enzyme/substrate ratio is $1:10^5$) is due to the progressive binding and cleavage of triple helical collagen I by a minute amount of enzyme molecules, which must undertake the turnover process several times to bring about an observable unwinding. As a whole, this set of observations represents clear evidence that binding of collagen I by MMP-2 is enough to induce the unwinding of the triple helical assembly, with a behavior completely different from that observed in the case of MMP-8[26] and ectMMP-14 (data not shown). The unwinding of the collagen I triple helix observed in Figure 5 is also confirmed by the fact that collagen I, which is still intact after 3 h of incubation at 37 °C with 2 μM fully inhibited MMP-2 (see Figure 1, lane 4), is instead more sensitive to trypsin degradation (see Figure 1, lane 5).

Ex vivo collagenolytic activity of MMP-2

To validate the powerful collagenolytic activity of MMP-2 we carried out a cleavage test model against Achilles mouse tendon, which is predominantly made of collagen I.[42] Notably, it turned out that MMP-2 (10 pM active enzyme) retains its collagenolytic activity even against intact fibers of collagen. The strong-banded fibrils and microfibrils displayed by the control samples show that the fibers examined possess a strong substructure (see Figure 6(a) and (b)). Figure 6(c) and (d) illustrates the micrographs referring to the fibers exposed overnight to MMP-2 activity. Experimental conditions of examined samples are comparable to those employed for the kinetic experiments, unequivocally demonstrating a relevant proteolytic activity of MMP-2 against intact fibers of collagen I.

Discussion

The mechanism of collagen I processing by different MMPs is crucial for understanding the remodeling of the extracellular matrix, because fibrillar collagen is a major component of the architecture[1] and its degradation is heavily involved in growth factor-induced angiogenesis.[43] Interestingly, the melting temperature (T_m) of collagens is

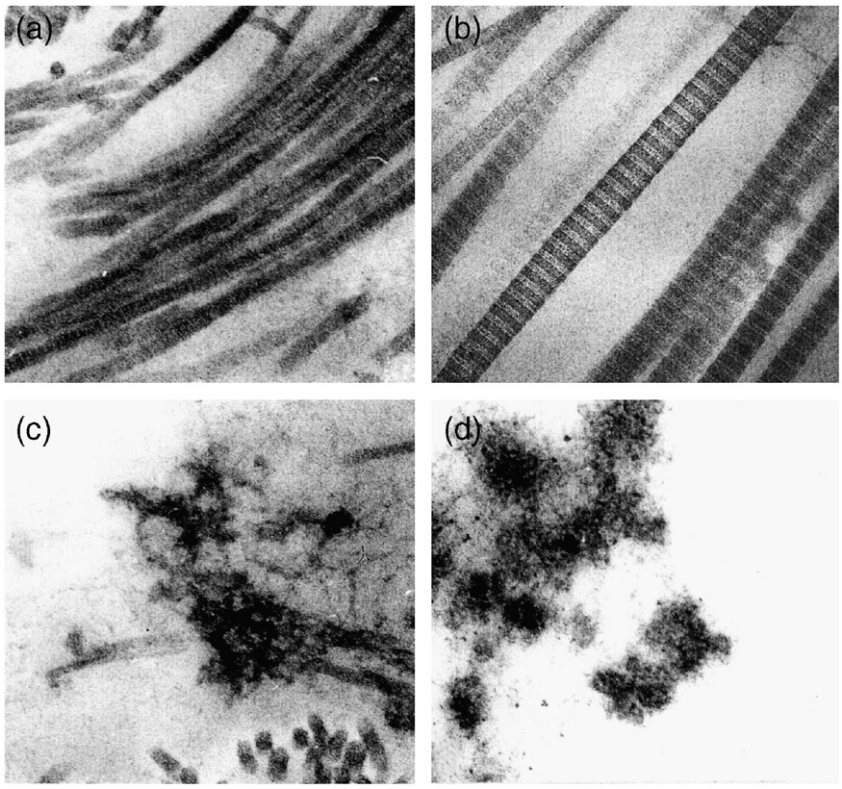

Figure 6. Transmission electron micrographs of the Achilles' tendons from 6-week-old mice. (a) and (b) Collagen fibers not exposed to the MMP-2 action; (c) and (d) collagen fibers treated with MMP-2. For (a) and (c) the optical magnification is ×28,500; for (b) and (d) the optical magnification is ×52,000. For further details, see the text.

related to the content of amino acids (i.e., proline and hydroxyproline). Thus, differences in the amino acidic content of two types of α chains (i.e., α-1 and α-2 chains)[44] should be reflected in different conformational stabilities within the triple helix, casting some light on the different mechanisms for the various MMPs.

Soluble fibrillar collagen I has been shown first to be enzymatically processed by collagenases (i.e., MMP-1, MMP-8, and MMP-13),[25,45] but also by gelatinase A MMP-2,[46] gelatinase B MMP-9,[47] and by MMP-14.[48] In particular, because MMP-14 is a membrane-bound MMP, its ectodomain (ectMMP-14) is also active on collagen I.[14] In any event, it is very important to outline that the present paper clearly shows that cleavage of native fibrillar collagen I by gelatinase A actually takes place in fibers from mouse tendon, as observed by electron microscopy (see Figure 6), reinforcing the idea that it may occur as well under *in vivo* conditions.

However, since the size of the triple helical collagen I is exceedingly large to be accommodated in the active site of either of these enzymes,[3,28–30] interaction and cleavage must occur through a series of steps, which allow the cleavable peptide bond to come into close contact with the active site. In this respect, it has been proposed that collagenases, such as MMP-1 and MMP-8, might interact with one chain at a time,[21,25] locally unwinding the triple helix, probably through the hemopexin-like domain, because its absence brings about a drastic change in the way collagen I is processed.[27] MMP-14 as well appears to interact with collagen I preferentially through the linker-hemopexin-like domain, which also seems crucial for the cleavage event,[23,49,50] even though the mechanism has been proposed to be somewhat different from that of collagenases.[23,51] On the other hand, even though a MMP-2 mutant (missing the fibronectin-like domain) retains the capability of cleaving collagen,[17] MMP-2 does not seem to interact with fibrillar collagen I through its hemopexin-like domain.[52] In fact, the fibronectin-like domain appears to be the preferential interaction site, probably inducing some conformational change, as from circular dichroism employing the CBD alone[23] and fully inhibited MMP-2 (see Figure 5). The driving force exerted by the CBD of MMP-2,[23,51] which is not present in other MMPs, raises the question of whether the mechanism of triple helicase activity of MMP-2 must be fundamentally different from that of collagenases.

The answer to this question comes from circular dichroism observations, where a drastic difference can be observed for the interaction of MMP-8 and ectMMP-14 with triple helical collagen I compared with MMP-2. Thus, no meaningful alteration of the triple helical assembly of collagen I is observed upon binding only with MMP-8[26] as well as with ectMMP-14 (see above), whereas a disruption of the triple helix assembly is observed upon the addition of MMP-2 fully inhibited by Batimastat (in an enzyme concentration-dependent fashion for comparable concentrations of substrate and enzyme; see Figure 5). These results clearly indicate that while binding of the hemopexin-like domain of MMP-8 or of ectMMP-14 does not bring about any substantial alteration of the triple helix (if no subsequent cleavage occurs), the interaction of MMP-2 alone through its fibronectin-like domain is sufficient to induce an extended and widespread unwinding of

fibrillar collagen I, even in the absence of any cleavage of the substrate. Furthermore, on the basis of the concentration of inhibited MMP-2 (i.e., 2 μM, which is enough only for binding to the α-1 chain; see K_m in Table 1), we can extend our interpretation, claiming that interaction of the fibronectin-like domain of MMP-2 with the α-1 chain of fibrillar collagen I is sufficient to induce an overall unwinding of a large portion of the triple helix. On the basis of such considerations and observations, the present data of MMP-2 suggest a preferential interaction of the fibronectin-like domain, which is not present in ectMMP-14 and MMP-8, with the α-1 chain of fibrillar collagen, a conclusion that is also in line with previous observations on the interaction of the whole fibronectin molecule with fibrillar collagen I.[53]

This difference among the three MMPs investigated for the collagen I triple helix binding mechanism is also reflected in the catalytic parameters (see Table 1), because MMP-2 interacts preferentially with the α-1 chain (as shown from the very low value for K_m; see Table 1), while it cleaves preferentially the α-2 chain (as shown from the much higher value of k_{cat}; see Table 1). Conversely, MMP-8 and ectMMP-14 appear instead to process the two types of chains of fibrillar collagen I in a similar fashion, without showing any preference for either chain (as shown from the values of K_m and k_{cat} for α-1 and α-2 chains; see Table 1). Therefore, these data seem to suggest that as long as the triple helix is maintained, no preferential enzymatic action is exerted on the two types of chains. On the other hand, whenever unwinding occurs, as with binding by MMP-2, the two types of chains display a drastically different propensity for proteolytic attack. The peculiar behavior of MMP-2 in processing the two types of chains of collagen is not surprising, because binding of the CBD (and thus likely also of the fibronectin-like domain) has been proposed (and is substantiated by the present data) to involve simultaneously two collagen chains,[54] exerting triple helicase activity.[15] The distinctive features of MMP-2 action on collagen I, which requires a preliminary unwinding of the triple helix, also account for the dramatic temperature dependence of MMP-2 cleavage activity on collagen I (which is almost vanishingly small at 25 °C).[45] Thus, unlike collagenases and ectMMP-14, the activation free energy for the enzymatic processing of the triple helical collagen I by MMP-2 includes (beside the intrinsic temperature dependence for the proteolytic activity)[37] a contribution from the unwinding of the triple helix of collagen I. As a matter of fact, the energy needed for the unwinding by MMP-2 is much higher at 25 °C (thus slowing down the process) than at 37 °C, where the triple helical arrangement is much looser. This observation is in line also with recent data on the enzymatic activity of several MMPs toward synthetic triple helical substrates,[24] where MMP-2 turned out to be less active than MMP-8 and MMP-14 toward tighter and more thermostable substrates.

As a whole, like at 25 °C,[20–24] at 37 °C a dramatic difference can be outlined between the proteolytic mechanism of MMP-8 (as a possible prototype of the collagenases action) and ectMMP-14 on one side and MMP-2 (as a possible prototype of gelatinases) on the other side, and this appears mostly related to the interaction mode with collagen I. Thus, MMP-8 and ectMMP-14 interact through the hemopexin-like domain, without significantly perturbing the triple helical assembly of fibrillar collagen, likely processing one chain at a time in a similar way (as shown by the closely similar catalytic parameter for the two chains; see Table 1). On the other hand, MMP-2 actually induces a denaturation of fibrillar collagen (likely binding the α-1 chains; see Table 1) and then it enzymatically processes fibrillar collagen, cleaving preferentially the α-2 chain (see Table 1) and suggesting that the unwinding induced by the binding of MMP-2 is possibly the rate-limiting step for the proteolytic attack of collagen I by MMP-2 (Figure 7).

Structural interpretation of the preferential enzymatic action on the α-2 chain by MMP-2 (see Table 1) is not straightforward, because we do not know the detailed structural characteristics of the two different chains. However, even though both amino acid sequences of type I collagen are homologous, the α-2 chain is more hydrophobic and has a lower amino acid content than the α-1 chain[1] and it has been shown that the higher amino acid content of α-1 chain gives a larger contribution to the conformational stability of the collagen triple helical structure.[4,55] Therefore, a purely speculative consideration might try to correlate the higher hydrophobicity and the lower amino acid content of α-2 chains to a higher value of K_m and a faster k_{cat} (see Table 1).

As a concluding remark, binding through the hemopexin-like domain (as for MMP-8 and ectMMP-14) brings about a topologically specific interaction, accompanied by a very limited and mild alteration of the triple helix assembly, and it underlies similar processing for the two types of chains forming the collagen triple helix. On the other hand, binding through the fibronectin-like domain (as for MMP-2) grossly perturbs the triple helical structure, leading to more extended and chain-specific substrate cleavage. These functional differences appear to reflect and can accounted for the distinct role of these MMPs "*in vivo*," which is tissue modeling for collagenases (such as MMP-8 and in part ectMMP-14), while gelatinases (such as MMP-2) seem to be more involved in disruptive events related to pathological processes.

Materials and Methods

Materials

Purity of MMP-2, MMP-8, and ectMMP-14 proenzymes, (R&D Systems, London, UK) wasbeen measured by SDS-PAGE described by Laemmli.[56] After the gels were run,

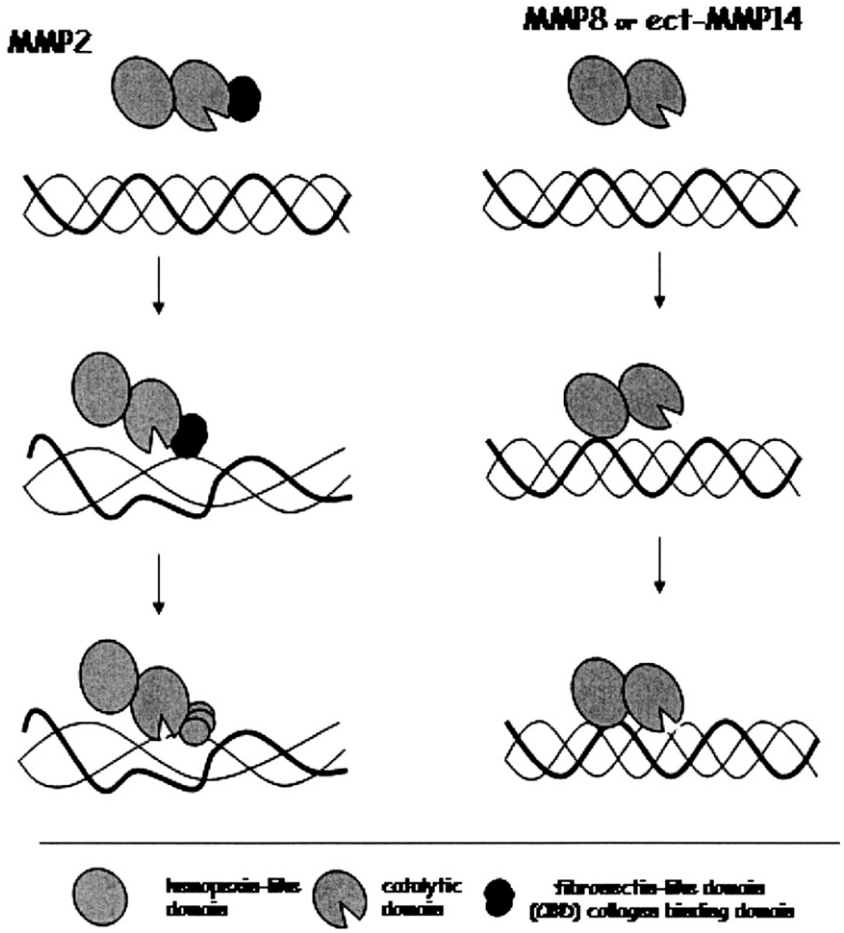

Figure 7. (Left) A molecular sketch of the possible steps for the cleavage of triple helical fibrillar collagen I by MMP-2, showing the interaction through the fibronectin-like domain and the unwinding of the triple helix before cleavage. (Right) A molecular sketch of the possible steps for the cleavage of triple helical fibrillar collagen I by MMP-8 and/or ectMMP-14, showing the interaction through the hemopexin-like domain, which leaves intact the triple helix before the cleavage. Thin lines refer to α-1 chains of the triple-helical collagen I and thick lines refer to the α-2 chain. For further details, see the text.

they were stained using a silver staining kit (Bio-Rad, Hercules, CA, USA). The broad spectrum protein markers (Bio-Rad) were used as molecular mass standards.

The stability of each MMP was checked by zymography (employing as substrates either gelatin and collagen), as follows: 2 μl of MMP in collagen solution was mixed with a 5-fold excess of sample buffer (0.25 M Tris-HCl, 0,8% SDS, 10% glycerol, and 0.05% bromophenol blue) and run on 12% SDS-PAGE containing either 1 mg/ml gelatin or bovine type I collagen as described previously.[56]

Trypsin^{tpch} and soybean trypsin inhibitor were purchased from Sigma (St. Louis, MO, USA). The quenched fluorogenic substrates employed for activity assays were (7-methoxycoumarin-4-yl)acetyl-Pro-cyclohexylalanine-Gly~*nor*valine-His-Ala-*N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl-$NH_2$and (7-methoxycoumarin-4-yl) acetyl–Pro-Leu-Gly~Leu-*N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl-Ala-Arg-NH_2, purchased from Calbiochem (La Jolla, CA, USA). Batimastat (BB-94), a peptidomimetic inhibitor, which stoichiometrically inhibits MMPs, was kindly provided by British Biotech Pharmaceutical (Cowley, Oxford, UK).

Acid-soluble calf collagen I, purchased from Sigma, was dissolved overnight at a concentration of 1 mg/ml in 0.1 M acetic acid solution at room temperature. Afterward, the suspension was centrifuged at 10,000 *g* for 1 h and the supernatant, containing the dissolved collagen, was used. We also used rat-tail tendon type I collagen, isolated by standard ammonium sulfate precipitation procedures from the tail tendons of 12-week-old Wistar rats, as previously described by others in detail.[52,57]

The amount of collagen has been quantified as described,[58] using soybean trypsin inhibitor as a reference. The triple helical nature of collagen has been checked by circular dichroism and confirmed by the absence of collagen degradation in trypsin control assays at an enzyme/substrate ratio of 1:10 over 3 h at 28 °C, as described previously.[23] In order to validate that collagen retains its properties, even after having been exposed to 37 °C for 24 h, we brought the sample back to 28 °C and repeated trypsin control assays: no degradation was observed under these conditions (see Figure 1, lane 2). Lyophilized collagen was stored at −80 °C, and stock solutions were prepared as needed. No significant difference in the triple helix content and stability was detected between the two preparations of collagen after solubilization.

Achilles' tendons, surgically extracted from young mice (manipulated as described by the ethical guidelines), were used for the electron microscopy experiments.

Methods

Enzymatic assays

Recombinant human MMP-2 and MMP-8 proenzymes were activated by incubating 0.1 mg/ml progelatinase solution with 0.25 mM of aminophenyl mercuric acid (Sigma) at 37 °C for 30 min. The activation of pro-ectMMP-14 was performed using 5 μg/ml trypsin^{tpch} at 25 °C. Trypsin activity was quenched using 50 μg/ml soybean trypsin inhibitor.

The concentration of activated MMP was determined by classic fluorimetric assay, as described,[59] following the

progressive decrease of hydrolysis (upon the addition of Batimastat (BB-94)) of the quenched fluorogenic substrate (7-methoxycoumarin-4-yl)acetyl-Pro-cyclohexylalanine-Gly~*nor*valine-His-Ala-*N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl-NH_2 (λ_{exc} = 325 nm, λ_{em} = 398 nm) for MMP-8 and (7-methoxycoumarin-4-yl)acetyl–Pro-Leu-Gly~Leu-*N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl-Ala-Arg-NH_2 (λ_{exc} = 325 nm, λ_{em} = 393 nm), which is resistant to trypsin action, for MMP-2 and ectMMP-14. All measurements were performed at 37 °C using a solution of 50 mM Tris-HCl, 0.1 M NaCl, 10 mM $CaCl_2$ plus 0.05% Brij 35 (polyoxyethylenelaurylether) buffered at pH 7.3.

Determination of kinetic and thermodynamic parameters

Kinetic analyses of α-chain degradation by MMP-2, MMP-8, and ectMMP-14 were performed in 50 mM Tris-HCl, 0.1 M NaCl, 10 mM $CaCl_2$, at pH 7.3, employing different concentrations of bovine collagen I (spanning between 0.3 and 3.2 μM). Activated MMP was then added to the solutions (10 pM final concentration). It is worth stressing that MMPs alone are not stable after prolonged incubation at 37 °C; however, their stability is significantly increased in the presence of the substrate, as also reported by others.[19,60]

For each collagen concentration, mixtures were kept at 37 °C and small aliquots were harvested at different time intervals. Reactions were stopped by the addition of SDS-PAGE loading buffer containing 20 mM EDTA and frozen to −80 °C until used. Aliquots in the reducing sample buffer were separated on 7.5 or SDS-(10%) PAGE gels and stained using either (i) the silver staining method or (ii) 0.5% Coommassie blue and then destained until substrate bands were clearly visible. The Coommassie blue electrophoretic spots, corresponding to different aliquots at different time intervals, were analyzed by a laser densitometer (LKB 2202 UltraScan) and their intensity was calibrated (to obtain concentration values) using standard substrate solutions. For each α-chain (i.e., α–1 and α–2 chains) the substrate disappearance rates were derived at each collagen concentration employed.

The initial velocities were measured within 1 h, that is the time period during which various MMPs were checked to be stable. Within this time interval, the degradation rate was constant and less than 10% of the substrate was degraded. This ensured a steady-state condition for the first cleavage step and was a prerequisite for the subsequent analysis step, which was carried out by simultaneous fitting of velocity experimental data described by both the Lineweaver-Burk equation (Equation 1(a)):

$$\frac{E_0}{v} = \frac{K_m}{k_{cat}}\frac{1}{[S]} + \frac{1}{k_{cat}} \tag{1a}$$

and the Eadie-Hofstee equation (Equation 1(b)):

$$\frac{v}{E_0} = k_{cat} - \frac{v}{E_0[S]}K_m \tag{1b}$$

where E_0 is the total enzyme concentration, v is the actual rate (expressed as mol/s), K_m is the Michaelis-Menten equilibrium constant (expressed as mol), k_{cat} is the rate-limiting step kinetic constant (expressed as s^{-1}), and [S] is the substrate concentration. This procedure allowed more reliable values for the k_{cat} and K_m parameters to be obtained.

Circular dichroism experiments

CD spectra were recorded on a Jasco J-710 spectropolarimeter equipped with a thermostated cell holder and connected to a data station for signal averaging and processing. All spectra are averages of four scans (unless otherwise specified; see Figure 4(b)) and were recorded using quartz cells of 2-mm pathlength.

For the spectroscopic observations bovine collagen I was entrapped in agarose gel by mixing the solubilized protein to a 1% low-melting-point agarose solution at pH 3.0 and 37 °C. After being stirred rapidly the mixture was poured on a simple gel casting (Mini-protean II, Bio-Rad) and immediately cooled to obtain the gel.[26] Final concentrations were 0.5% for agarose and 1 μM for collagen. The thickness of the homogeneous gel was 1 mm.

As a starting control, CD measurements were performed on samples of agarose gel soaked in sample buffer, which did not show any ellipticity, and data obtained were considered background. In addition, control CD measurements were also performed using gel-entrapped collagen at pH 3.0, as well as solubilized collagen I at the same pH and concentration conditions: no spectral difference between them was detected over the 200 to 250-nm spectroscopic range (a spectral region representative of the triple helical arrangement of collagen I)[61] (data not shown), confirming that the collagen conformation was not altered by the gel entrapment procedure. This was also tested by incubating the agarose gel-entrapped collagen with 10 nM trypsin for 3 h at 37 °C: no evidence of degradation was detected, as indicated by the lack of ellipticity changes at 223 nm over the incubation period (data not shown).

For kinetic experiments, slices of suitable size were cut from the homogeneous gel and kept overnight in buffer solution to reach the desired pH without the formation of aggregates and then used for CD measurements.[26] Because collagen I has been reported to be somewhat thermally unstable in solution,[34] gel-entrapped collagen samples of each proteolytic assay were monitored for as long as 3 days at 37 °C and no significant change in CD spectra was detected in the absence of the enzyme (data not shown). The CD spectra so obtained are in agreement with those obtained for collagen-like peptides showing the characteristic triple helix maximum in the 223 to 225-nm range.[20] Therefore, we achieve a "quasi-native" model for evaluating structural arrangements during proteolytic processes on type I collagen.[26] The concentrations of the agarose gel-entrapped collagen I were chosen so that the recorded CD spectra were obtained under conditions comparable to those employed for the fragmentations of soluble collagen I.

The kinetics of the unwinding of the triple helix was obtained in 0.2 M Tris-HCl, 1 M NaCl, 10 mM $CaCl_2$ solution at 37 °C, pH 7.3, by recording the spectroscopic range (200–250 nm) as a function of time of incubation with the enzyme. Therefore, we derived the time evolution of the unwinding process reporting the molar ellipticity at 223-nm values of CD spectra recorded in the presence of different concentrations of each MMP species (spanning from 2 nM to 2 μM).

As a control experiment, spectra of MMP (at the same concentration as that employed in the experiment) were recorded before every experiment in the same buffer solution. For all cases MMP ellipticity was vanishingly small (data not shown), ruling out the possibility of a mutual spectral distortion.

Electron microscopy

Achilles' tendons surgically extracted from 6-week-old mice were rinsed in phosphate-buffered saline and incubated overnight with active MMP-2 at 37 °C. Light

microscopy and ultrastructural analysis were performed on samples fixed in 25% glutarhaldehyde in phosphate-buffered saline at pH 7.4 and processed for transmission electron microscopy, as previously described.[62] Two independent observers evaluated three different experiments.

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