

IMMUNOHISTOCHEMICAL PROFILE OF VEGF, PGE₂ AND TGF- β IN INFLAMMATORY TENOSYNOVITIS OF CARPAL TUNNEL SYNDROME

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Inflammatory tenosynovitis is an inflammation that involves the tendons and synovial sheaths caused by minor trauma repeated for a long period of time. This inflammatory disease may be involved in the onset of tunnel carpal syndrome (CTS), because of the thickening of the tendon sheath that may produce an increase in the carpal canal pressure and damage of the median nerve in the wrist. Recent studies suggest that in patients with CTS pathological changes occur in the subsynovial connective tissue, such as vascular proliferation and non-inflammatory synovial fibrosis. However, little is known about the pathological mechanism of tenosynovial thickening. The aim of this study is to evaluate the potential role of vascular endothelial growth factor (VEGF), prostaglandin E₂ (PGE₂) and transforming growth factor-beta (TGF- β) in the modifications of connective synovial tissue of CTS specimens in order to determine whether these factors play a role in the development of this disease. Ten specimens from patients with CTS and four control tissues (cadavers) were analyzed by immunohistochemistry using specific antibodies against these growth factors. A temporary increase in the production of these molecules was found in cells within the vessels and synovial lining during the intermediate phase of the syndrome, when the histology of the tenosynovium changes from oedematous to fibrotic. Our data confirm a close correlation between the expression of PGE₂ and VEGF. Recent histological examinations have shown a marked increase in vascular proliferation and reduction of fibroblast density in specimens from CTS patients during the intermediate phase. Our study indicates that the expression of TGF- β in fibroblasts and vascular endothelial cells of synovial connective tissues of CTS patients was significantly higher than in those of controls. These findings suggest that angiogenesis appears to take place as a part of a regenerative reaction that results in fibrosis. We believe that VEGF, TGF- β and PGE₂ may be potential therapeutic targets in the treatment of this disease although proof of this evidence requires further studies.

Carpal tunnel syndrome (CTS) is an extremely common entrapment neuropathy resulting from

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compression of the distal median nerve within the carpal tunnel, located in the proximal part of the palm of the hand (1). Recent studies suggest that, in patients with CTS, pathological changes occurs in the subsynovial connective tissue (SSCT) (2). Tenosynovial thickening may cause an increase in canal pressure and damage to the median nerve in the wrist. However, the causes of such events still remain unclear. Inflammatory tenosynovitis, inflammation of the tendon and tendon sheath, is an uncommon cause of median nerve compression at the wrist. Biopsy specimens taken during open carpal tunnel surgery confirm that inflammation is very rare and that oedema and non-specific fibrosis are the most common histological findings. Scelsi et al. (3) correlated the histological changes with clinical history and found that early CTS presents with a thickened oedematous synovial sheath, while advance CTS presents with extensive fibrosis associated with significant type III collagen deposition. The symptoms of CTS change with progression of the disease. In the early phase, patients complain of intermittent pain, numbness, and tingling, while median nerve function is well preserved. In the intermediate phase, the symptoms become constant, and median nerve dysfunction becomes apparent. In the late phase, patients show severe motor and sensory disturbances, but they often complain of less pain than in the early phase (4). Pro-inflammatory cytokines are considered to play important roles in the initiation and development of joint diseases, including osteoarthritis (OA) and rheumatoid arthritis (RA) (5-7). IL-1 and tumour necrosis factor- α (TNF- α) are the most prominent cytokines for developing synovial inflammation and play a predominant role in the etiopathology of joint disease. It has been established that IL-1 enhances synovial fibroblast DNA synthesis (8) and activates synoviocytes to secrete soluble mediators such as collagenase (9), PGE₂ (9), IL-6 (10), and VEGF (11). These mediators are involved in inflammatory response, joint destruction and angiogenesis. The physiological and pharmacological actions of PGE₂ on cell growth and function are mediated by a specific group of seven transmembrane receptors. PGE₂ receptors have been classified into four subtypes, designated the EP1, EP2, EP3, and EP4 receptors (12, 13). EP1 receptors are associated with phospholipase C and phosphoinositol turnover and

stimulate the release of intracellular calcium. EP2 and EP4 receptors increase cyclic AMP levels via activation of adenylate cyclase, whereas EP3 receptor variants mediate multiple signal pathways such as inhibition or stimulation of cyclic AMP levels, activation of phospholipase C, and mobilization of intracellular calcium. Some authors have reported that inflammatory cells are rare and that edema and nonspecific fibrosis are the most common histological findings of tenosynovium in the CTS (14). Freeland et al. (15) suggested that ischemia-reperfusion injury is an important cause of idiopathic CTS. This type of injury appears to increase the levels of several types of proinflammatory cytokines in the tenosynovium and possibly causes the pathologic changes observed in idiopathic CTS. The purpose of our study was to investigate the role of vascular endothelial growth factor (VEGF), prostaglandin E₂ (PGE₂) and transforming growth factor- β (TGF- β) in the subsynovial connective tissue swelling that occurs within the carpal tunnel. In the tenosynovial of CTS patients, VEGF and PGE₂ regulate not only angiogenesis but also vascular permeability (16). VEGF is produced by the synovial lining cell layer and vascular smooth muscle cells (16), following binding of PGE₂ to its EP2 receptor. A significant increase in TGF- β receptor 1 expression occurs in the fibroblasts of CTS patients compared with unaffected individuals, and collagen III is significantly more abundant in CTS patients than in controls (17). TGF- β plays a vital role in mediating extracellular matrix deposition and remodeling, which occur in tissue repair and fibrosis (18, 19). We hypothesized that VEGF, in coordination with PGE₂, is involved in vascular lesions and thickening of the synovial lining. However, these two molecules appear to play a minor role in oedema formation and onset of CTS because their level is low during the early phase of CTS. In addition, we found that the percentages of TGF- β expression were less in the control synovial tissue fibroblasts than in the CTS patients.

MATERIALS AND METHODS

Ethical considerations

The study group included 10 cases of surgically excised subsynovial connective tissues of patients suffering from CTS (2 patients in early phase of CTS and 8 patients in intermediate phase of CTS) aged 45-80 years, together

with 4 autoptic specimens harvested as control cases. Cadaver specimens were harvested from four cases: three cases of mangled body and one of beheading. In these cases, biopsies may be performed within 24 hours after the death. During excision of subsynovial tissues of patients suffering from CTS, apart from topical anaesthesia, no other chemical product or pharmaceutical drug was administered. Experiments were performed in compliance with the Italian laws and guidelines concerning the patients informed consent. The ethics committee of the Hospital approved our study according to the European Community and Italian laws.

Immunohistochemical analysis

Control morphological sections were stained with hematoxylin-eosin. The following molecules were investigated: vascular endothelial growth factor (VEGF), prostaglandin E₂ (PGE₂) and transforming growth factor- β (TGF- β).

Small fragments of the tenosynovium from the CTS patients' samples were washed in PBS, fixed in 10% formalin and embedded in paraffin. The method employed for immunohistochemical tests was ABC/HRP technique (avidin complexed with biotinylated peroxidase). Serial 3- μ m thick sections were cut using a rotative microtome, mounted on gelatin-coated slides and processed for immunohistochemistry. These sections were deparaffinized in xylene and dehydrated and were immersed in citrate buffer (pH 6) and subjected to microwave irradiation twice for 5 minutes (750 watts). Subsequently, all sections were treated for 30 minutes with 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. To block non-specific binding, the slides were incubated in 3% normal goat serum in PBS for 30 minutes at room temperature. The slides were incubated overnight at 4°C with primary mouse monoclonal antibodies against human VEGF-A diluted 1/100 (abcam Cambridge Science Park, UK, ab1316), against human TGF- β 1 diluted 1/100 (abcam Cambridge Science Park, UK, ab49574) and with primary rabbit polyclonal antibody against human PGE₂ diluted 1/500 (Abcam Cambridge Science Park, UK, ab2318). Optimal antisera dilutions and incubation times were assessed in a series of preliminary experiments. After exposure to the primary antibodies, slides were rinsed twice in phosphate buffer and incubated for 1 h at room temperature with the appropriate secondary biotinylated goat anti-mouse or anti-rabbit IgG (vector laboratories Burlingame, CA, USA, BA9200 and BA1000) and with peroxidase-conjugated avidin (Vector laboratories, Burlingame, CA, USA, Vectastain Elite ABC Kit Standard* PK 6-100) for 30 minutes. After a further wash with phosphate buffer, slides were treated with 0.05% 3,3-diaminobenzidine (DAB) and 0.1% H₂O₂. Finally,

sections were counterstained with Mayer's hematoxylin and observed using a light microscope. Negative control experiments were carried out: i) by omitting the primary antibody; ii) by substituting the primary antibody with an equivalent amount of non-specific immunoglobulins; iii) by pre-incubating the primary antibody with the specific blocking peptide (antigen/antibody = 5 according to the supplier's instructions). The staining assessment was made by two experienced observers in light microscopy. We assessed the immunoreactivity for VEGF, TGF- β and PGE₂ in the synovial tissue, mainly observing the extracellular matrix, vascular endothelium and vascular smooth muscle cells. The intensity of the immune reaction was assessed microdensitometrically using an IAS 2000 image analyzer (Delta Sistemi, Rome, Italy) connected via a TV camera to the microscope. The system was calibrated taking the background obtained in sections exposed to non-immune serum as zero. Ten 100 μ m² areas were delineated in each section by a measuring the diaphragm.

Statistical analysis

Quantitative data of the intensity of the immune staining were analyzed statistically by analysis of the variance (ANOVA) followed by Duncan's multiple range test as a post hoc test. The comparison of the expression levels of VEGF, TGF- β and PGE₂ in the tenosynovium from CTS and normal patients was carried out by *t*-test. Statistical analyses were performed using the SPSS statistical software package version 12.0. The results were considered as statistically significant when P-value<0.05.

RESULTS

Fragments of tenosynovium from CTS patients treated with surgical excision were examined under light microscope. Immunolabelling of type III collagen demonstrated that marked extracellular matrix remodelling occurs in the tenosynovium with disease progression (data not shown). In the early phase of carpal tunnel syndrome, the synovial connective tissue was oedema, as shown in recent studies by the characteristic findings of connective tissue separation and villous thickening of the tenosynovium. At this stage, type III collagen expression was weak while the collagen bundles were mostly composed of type I collagen. Specimens from the intermediate phase of CTS clearly showed disorganization of collagen bundles characterized by thickening, destruction, and hyaline degeneration with marked reduction of the fibroblast density. At

Table I. Results of immunohistochemical analysis for VEGF, TGF- β and PGE₂ in normal synovial connective tissue.

	EXTRACELLULAR MATRIX	VASCULAR ENDOTHELIUM	VASCULAR SMOOTH MUSCLE
VEGF	+	+	+/-
PGE ₂	-	-	-
TGF- β	+/-	-	-

+++ : strong immunoreactivity; ++ : relevant immunoreactivity; + : moderate immunoreactivity; +/- : weak immunoreactivity; - : absence of immunoreactivity.

Table II. Results of immunohistochemical analysis for VEGF, TGF- β and PGE₂ in the intermediate phase of CTS.

	EXTRACELLULAR MATRIX	VASCULAR ENDOTHELIUM	VASCULAR SMOOTH MUSCLE
VEGF	+++	++	++
PGE ₂	++	++	++
TGF- β	+	+	+

+++ : strong immunoreactivity; ++ : relevant immunoreactivity; + : moderate immunoreactivity; +/- : weak immunoreactivity; - : absence of immunoreactivity.

this phase of disease progression, immunolabelling of type III collagen demonstrated that marked collagen remodelling had taken place insidiously between the early and the late phases, because the type III collagen was predominant in this stage (data not shown). Previous studies have shown that the fibroblasts produce ground substances such as proinflammatory cytokines and matrix metalloproteinases during the intermediate phase of syndrome. These data show that ECM remodelling and synovial connective tissue thickening take place during the intermediate phase (14). The normal synovial tissue showed the normal vascular architecture of vessels, in contrast to arteries in the tenosynovium of CTS patients during intermediate phase, that presented intimal thickening and, in some cases, occlusion of vessels by thrombi. In the current study we investigated the expression of VEGF, PGE₂ and TGF- β in specimens from intermediate phase of CTS in comparison with control tissues. Our study showed relevant immunoreactivity of VEGF and PGE₂ in the synovial lining, vascular smooth muscle and endothelium of CTS patients in the intermediate phase (Figs. 1, 2). The normal synovial tissue

presented a moderate expression level for VEGF, demonstrating a statistically significant difference in comparison with the intermediate phase of the disease (Fig. 3). In contrast to this pathological tissue, no PGE₂ immunoreactivity was observed in the control specimens (Fig. 4). Furthermore, the expression level of VEGF and PGE₂ varied from one area to another, indicating that the tenosynovial reaction did not occur uniformly. Another remarkable finding is that VEGF expression was very weak in the early phase of CTS (Fig. 5). These data indicates that, although VEGF is also a known vascular permeability factor and has been shown to be involved in brain oedema, it does not appear to participate in CTS tenosynovial oedema. Statistical analysis of VEGF and PGE₂ immunolabeling distribution shows a significant correlation between VEGF and PGE₂ levels. This indicates that PGE₂ may be functionally linked to VEGF and may contribute to tenosynovial changes in CTS. TGF- β immunolabeling was very weak in the fibroblasts of normal connective tissue (Fig. 6), in contrast to that of the intermediate phase of CTS, during which it appears to be moderately expressed in vessel endothelial cells and in the fibroblasts

Table III. Levels of VEGF, TGF- β and PGE₂ examined in CTS and control specimens, and respective levels of statistical significance (t-test).

	THE INTERMEDIATE PHASE OF CTS (8 PATIENTS: 1 specimen for each patient)	CONTROLS (4 PATIENTS: 1 specimen for each patient)	P-VALUE
VEGF			
Extracellular matrix cells	85.52%	49.80%	P=0.0001
Vascular endothelial cells	75.02%	52.30%	P=0.0015
Smooth muscle cells	74.02%	17.5%	P=0.0001
PGE ₂			
Extracellular matrix cells	71.12%	6.3%	P=0.0000
Vascular endothelial cells	66.12%	4.3%	P=0.0000
Smooth muscle cells	64.12%	2.55%	P=0.0000
TGF- β			
Extracellular matrix cells	54.86%	20.05%	P=0.0040
Vascular endothelial cells	55.76%	7.05%	P=0.0002
Smooth muscle cells	57.26%	5.55%	P=0.0001

of subsynovial connective tissue (Fig. 7). These findings support the hypothesis that oxidative stress in subsynovial connective tissue caused by repeated transient ischemia-reperfusion injury is associated with pathological changes of CTS. Various cytokines and growth factors are involved in the development of fibrosis, but TGF- β is considered to be the most potent profibrogenic cytokine. In fact, oxidative stress in subsynovial connective tissue triggers cytokine production in fibroblast and endothelial cells and causes vascular hypertrophy and subsequent tissue fibrosis. The intensity of staining for VEGF, TGF- β and PGE₂ in human normal and CTS tenosynovium is presented in Tables I and II. The percentage values of growth factors-positive cells and P-values are shown in Table III.

DISCUSSION

The most important pathological event which characterizes carpal tunnel syndrome is the proliferation of stromal components in the subsynovial connective tissue. The stroma include vascular endothelium, activated fibroblasts and

matrix proteins. The formation of new blood vessels is of remarkable importance in many physiological and pathological processes such as tumor growth, wound healing and neovascularization of ischemic tissue, in which VEGF plays a main role (20). It is likely that in the subsynovial fibrosis of CTS, a mechanism of ischemia-reperfusion acts as the trigger for neo-angiogenesis (21). The possible involvement of angiogenesis in rheumatoid arthritis and osteoarthritis has been suggested for some time (22-24). Although treatments targeting VEGF or its receptor have been shown to be effective in the pathogenesis of arthritis, these data in the carpal tunnel syndrome are still unclear. It has been proposed that PGE₂ regulates the production of a diverse group of molecules, such as VEGF (25). Our study shows a close association between the expression levels in PGE₂ and VEGF, because PGE₂ may regulate VEGF production in the tenosynovium of CTS patients. Previous studies have made it possible to hypothesize that PGE₂ is induced by ischemia in the carpal tunnel which, in turn, causes oedema by increasing vascular permeability. Although we do not deny the role of ischemia in the pathophysiology

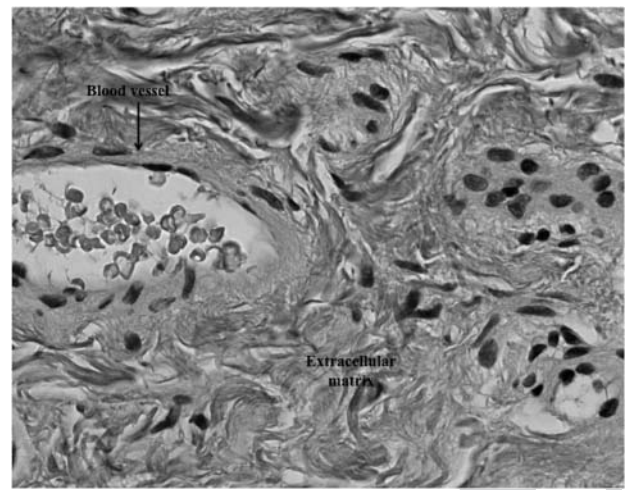
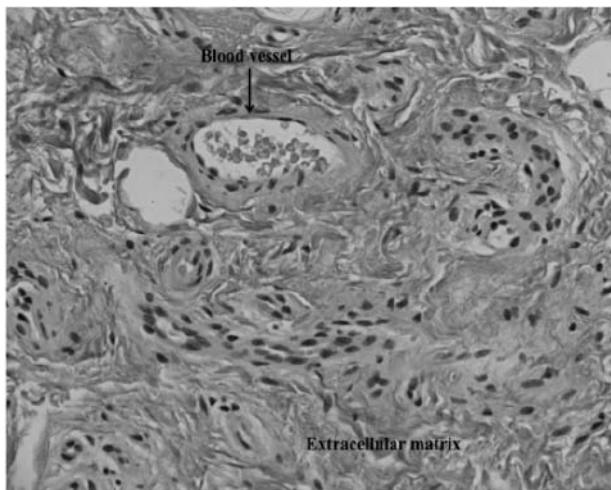


Fig. 1. Immunohistochemical analysis of specimens from intermediate phase of CTS for VEGF. VEGF immunoreactivity appears to be strongly positive in the stromal tissue, endothelial cells and vascular smooth muscle of blood vessels. **A** (20X) and **B** (40X).

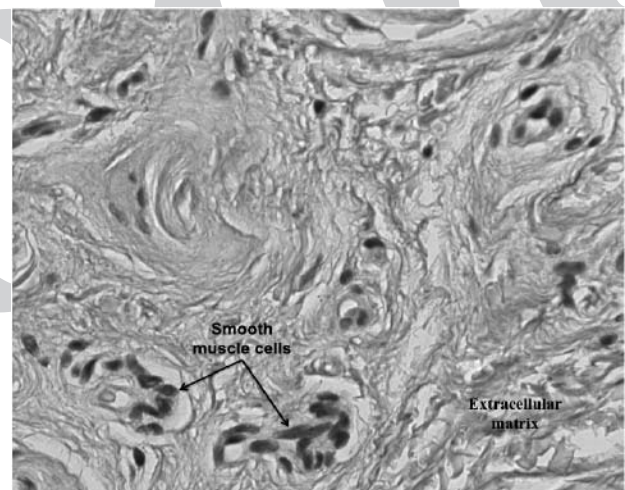
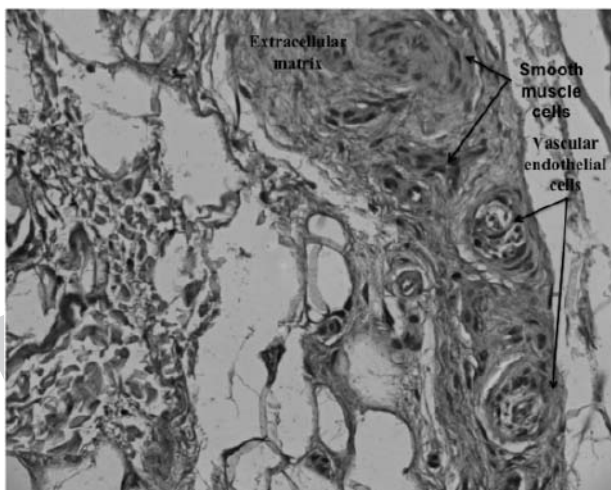


Fig. 2. Immunohistochemical analysis of specimens from the intermediate phase of CTS for PGE₂. PGE₂ immunoreactivity appears to be strongly positive in the stromal tissue, endothelial cells and vascular smooth muscle of blood vessels. **A** (20X) and **B** (40X).

Fig. 3. Immunohistochemical analysis of normal synovial tissue for VEGF. A moderate expression of VEGF is seen in the smooth muscle cells and extracellular matrix (40X).

of CTS, we feel at odds with this hypothesis regarding the role of PGE₂. In fact, the present study demonstrates that PGE₂ production increases during the intermediate phase of CTS, causing increased vascularity, vessel hypertrophy and tenosynovial thickening. Therefore, neither PGE₂ nor VEGF plays a role in oedema formation or onset of CTS. In view of the fact that their expression peaks occur during the intermediate phase, when ECM of synovial

connective tissue changes from oedema to fibrosis, PGE₂ and VEGF appear to contribute to tissue remodelling by elaborating new vessels. However, the regenerative attempt fails and results in fibrosis. In the subsynovial connective tissue examined, VEGF had a high expression in several types of cells with paracrine and autocrine action on the endothelial elements. These new data indicate that anti-VEGF therapies are potentially effective in many

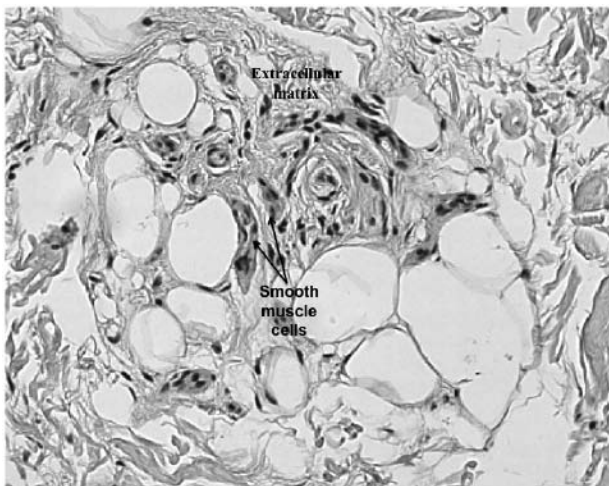


Fig. 4 Immunohistochemical analysis of human normal synovial tissue for PGE2. PGE2 expression was not found in the smooth muscle cells and extracellular matrix (20X).



Fig. 6. Immunohistochemical analysis of human normal synovial tissue for TGF- β . TGF- β expression was very weak in fibroblasts from normal synovial connective tissue (40X).

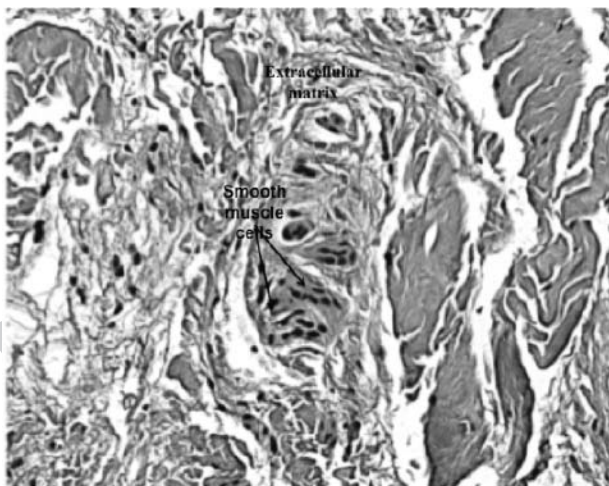


Fig. 5. Immunohistochemical analysis of specimens from CTS patients in the early phase for VEGF. VEGF immunoreactivity appears to be weakly positive in the stromal tissue, endothelial cells and vascular smooth muscle of blood vessels (20X).

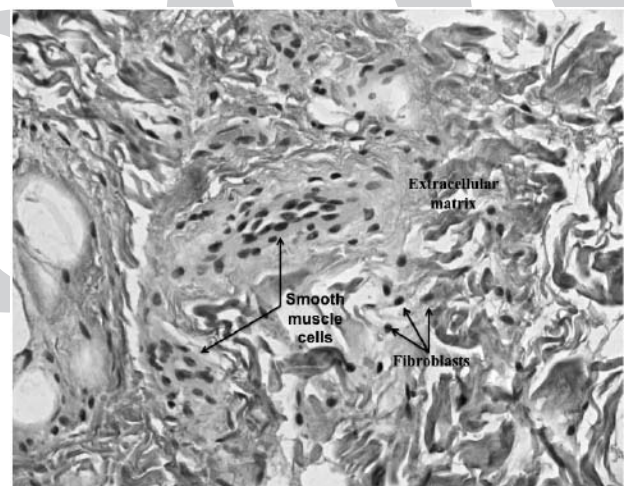


Fig. 7. Immunohistochemical analysis of specimens from the intermediate phase of CTS for TGF- β . TGF- β immunoreactivity appears to be moderately expressed in the smooth muscle cells and in fibroblasts of synovial connective tissue (40X).

pathological conditions (26, 27). Therefore, in future, administration of anti-VEGF drugs, could permit a non-surgical control of the pathological processes in the subsynovial tissue in patients suffering from CTS. The present study demonstrates that ischemia triggers a proliferative anomalous process not only for the vascular component, but also for stromal cells. In addition to the activation of fibroblasts and degeneration of type I collagen fibres, increased

synthesis of type III collagen, glycosaminoglycan, cytokines, neuropeptides and prostaglandins have been reported in the tenosynovium (28-32): this determines remodelling of the synovial connective tissue in the intermediate phase of CTS. Various cytokines and growth factors are involved in the development of fibrosis. TGF- β induce fibrosis via the stimulation of fibroblast activation (33), mediating extracellular matrix deposition and

remodeling. TGF- β increase ROS (reactive oxygen species) production in endothelial cells, epithelial cells, smooth muscle cells and fibroblasts (34-36). TGF- β increases ROS levels by suppressing the expression of several antioxidant enzymes (34, 37). Previous study demonstrated that oxidative stress in subsynovial connective tissue, caused by repeated transient ischemia-reperfusion injury, is associated with pathologic changes of CTS (35). Our study confirms this hypothesis by showing a significant increase in TGF- β expression in tenosynovium of CTS patients than in controls. These findings support the hypothesis that oxidative stress in subsynovial connective tissue triggers cytokine production in fibroblasts and endothelial cells, causing vascular hypertrophy and subsequent tissue fibrosis. To summarize, we present evidence that oxidative stress in the tenosynovium is associated with the pathogenesis of the carpal tunnel syndrome. In conclusion, our work confirms the recent observations about pathological changes in the CTS and supply new data concerning the cellular component of non-inflammatory fibrosis of tenosynovium. On the basis of such data, we believe that VEGF, PGE₂ and TGF- β may be potential therapeutic targets in the treatment of carpal tunnel syndrome. We conclude that the development of synthetic inhibitors of growth factors for therapeutic intervention could potentially allow a reduction of relapse rate and a non-surgical management of the CTS. Additional clinical and experimental investigations appear to be necessary to better clarify the biological role of these molecules in the development and progression of this type of syndrome.

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