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Mini Review

Redox Control of Apoptosis: An Update

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ABSTRACT

The redox environment of the cell is currently thought to be extremely important to control cell growth, differentiation, and apoptosis as many redox-sensitive proteins characterize these networks. A recent, widely accepted theory is that free radicals are not only dangerous species but, at low concentration, they have been designed by evolution to participate in the maintenance of cellular redox (reduction/oxidation) homeostasis. This notion derives from the evidence that cells constantly generate free radicals both as waste products of aerobic metabolism and in response to a large variety of stimuli. Free radicals, once produced, provoked cellular responses (redox regulation) against oxidative stress transducing the signals to maintain the cellular redox balance. Growing evidence suggests that in many instances the production of radical species is tightly regulated and their downstream targets are very specific, indicating that reactive oxygen species and reactive nitrogen species actively participate in several cell-signalling pathways as physiological "second messengers." In this review, we provide a general overview and novel insights into the redox-dependent pathways involved in programmed cell death. *Antioxid. Redox Signal.* 8, 2187–2192.

INTRODUCTION

THE PHENOMENAL RATE of expansion in the field of redox-regulated mechanisms controlling apoptosis justifies the revision of the forum issue assembled 2 years ago. In this short time we realized that, although the forum issue provided a general overview and novel insights into the redox-dependent pathways involved in programmed cell death, it is already outdated due to the remarkable progress that have been made in this field of research. Actually, even though the pathways implicated in the induction/execution of apoptosis have remained largely unaltered, many new pieces of the puzzle have been added during these years, thus rendering the redox signaling network, upstream of cell death processes, of more general application and controlled at multiple levels. In this brief overview, we will consider the progress made in the field from the published issue of March 2005, trying to take into account each step of apoptosis in which redox unbalance has been suggested to have a key role in activating/inhibiting the death program, starting from the binding of ligands to their cognate death receptors, along with the activation of downstream protein targets (protein kinases and transcription factors). The nature of oxidative stimuli together with the involvement of mitochondria and mitochondria-associated proteins in the cell responses will be also evaluated.

REDOX-REGULATED RESPONSE TO CELL SURFACE RECEPTORS ENGAGEMENT

The involvement of redox unbalance, especially that due to reactive oxygen species (ROS) production, has been exhaustively demonstrated to occur during the recognition of specific ligands by cell death receptors (5, 55). Moreover, the resulting ROS flux was frequently referred to as the early event associated with the activation of cell signaling downstream of receptor engagement (9, 41). Depending on the cell type and the protein machinery recruited, redox unbalance is able to induce a bifurcated signaling system, which could result in opposite fates: apoptotic death or antiapoptotic response. Two years ago Matsuzawa and Ichijo addressed the role of ROS and intracel-

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lular redox changes in the activation of mitogen activated protein (MAP) kinases (37). Recently they have elegantly demonstrated the role of the apoptosis signal-regulating kinase 1 (ASK1) in the induction of apoptosis under several conditions. Specifically, through the use of ASK1$^{-/-}$ mice, they have added novel findings on the independent- or receptor-associated redox-dependent activation of ASK1. Current results suggest a crucial role for ASK1 also in the activation of interferon (IFN) regulatory factor 3 (IRF3) (13) and the innate immune response to bacterial lipopolysaccharides (LPS) mediated by Toll-like receptor 4 (TLR4). In particular, Ichijo *et al.* (38) found that ASK1 is required for LPS-induced sustained activation of p38^{MAPK}, but not c-Jun N-terminal kinase (JNK) or NF-κB, by means of the formation of a complex with the adaptor molecule TNF receptor-associated factor 6 (TRAF6). In the pathway drawn, the role of the ASK1 inhibitory partner, thioredoxin (Trx), seems to be crucial as, by means of redox-sensitive vicinal thiols, it is oxidized to an intramolecular disulfide bridge-containing protein, changed in its structure and dissociated from ASK1, thus leading the phospho-signal to be propagated (29). By treating the cells with the flavoenzyme inhibitor dinitrophenyl iodonium or by interfering with translation of NADPH-dependent oxidase 4 (NOX4) mRNA, ROS were no longer produced, thus identifying NOX4 as the principal source of ROS responsible for phospho-signal transduction downstream of TLR4 (13). Moreover, Noguchi and co-workers demonstrated that endogenous ASK1 constitutively forms a high molecular mass complex named *ASK1 signalosome* of approximately 1,500–2,000 kDa, which also includes Trx (42). Upon ROS burst, such as after hydrogen peroxide (H_2O_2) treatment, this complex undergoes further molecular weight increase, due, at least in part, to the recruitment of TRAF6 and TRAF2. Since the latter protein has been previously described to be involved in mitochondrial-derived ROS output downstream of TNF-α binding, its recruitment to ASK1 signalosome indicates a more complicated model of regulation for ASK1-mediated signaling pathways (28, 55). This evidence could imply the existence of a novel and common signaling complex, downstream surface receptors; however, the specificity of cell response (apoptosis, cell survival, differentiation, and cytokine production) should rely on the different protein/protein interactions (ASK1/TRAF2 *versus* ASK1/TRAF6) at the intracellular portion of such receptors, upstream of ASK1 signalosome.

Chemical or genetic manipulation of ligand/receptor system could represent a good tool to modulate downstream cell responses: this suggestion becomes of more significance if we consider that dysfunction of the pathways controlled by such a system seems to concur in the etiology of many severe human diseases, such as cancer.

Although the binding of ligand to its death receptor usually should result in cell death by apoptosis, this process concomitantly could produce a pro-survival cell response mostly mediated by tumor necrosis factor/nuclear factor-κB (NF-κB). In this regard, 2 years ago Aggarwal and colleagues reported a detailed review on the involvement of TNF/NF-κB-mediated pathways in cell survival (48). Recently this group has made great progress to fully characterize the NF-κB-mediated signaling pathway by isolating and testing the inhibitory activity of several plant-derived natural compounds (1). Among these, curcumin (2, 3), and many other polyphenol derivatives (31, 32, 33, 46, 49, 52, 53, 54) have been found to inhibit NF-κB pathway in several tumor cell lines and at different levels, from IκB phosphorylation to the expression of NF-κB-regulated gene products. These novel findings could be of great importance in cancer prevention and therapy (27), especially in light of the latest evidence that demonstrates a pivotal role for NAD(P)H:quinone oxidoreductase 1 (NQO1) in producing ROS downstream of TNF engagement, in keratinocytes from NQO1$^{-/-}$ mice (4). The genetic deletion of NQO1 used in this study abrogates the phosphorylative cascade mediated by MEKK1, which concomitantly activates IκB kinase (IKK)/NF-κB signaling axis and all the members of the MAP kinase family: JNK, p38^{MAPK}, and p42/44 extracellular signal-regulated kinase 1/2 (ERK1/2) (4). The inhibition of the phosphorylative cascade is able to completely prevent MEKK1-dependent transient induction of MAP kinase members and the pro-survival effect of NF-κB, thus allowing the TRAF2-dependent signal to be propagated and caspase-dependent apoptotic program to be executed.

Overall, the observations from recent progress let us speculate that the site of production of ROS (mitochondria, NOX4, NQO1, or other NADPH oxidases) might represent a selective checkpoint in discriminating which response will be activated by the cell. Therefore, the activation of specific ROS-producing enzymes could be the determinant of the different histotype cell response downstream of surface receptor engagement.

REDOX-RESPONSIVE ANTI-APOPTOTIC PROTEINS: KEY COMPONENTS OF APOPTOTIC MACHINERY

In the Forum issue, great importance was given to the anti-apoptotic equipment exploited by the cell to counteract pro-apoptotic redox stimuli (23, 60). In this context, of particular importance is the evidence that an X-linked inhibitor of apoptosis (XIAP), known primarily for its caspase inhibitory properties, plays a role in copper metabolism (40). Indeed, it has been demonstrated that XIAP efficiently binds copper, a process that accelerates its degradation and decreases its ability to inhibit caspase-3. This phenomenon seems to be causative of the neurodegeneration observed in diseases related with copper toxicosis such as Wilson's disease. The anti-apoptotic role of XIAP is physiologically antagonized by its inhibitor, XIAP-associated factor 1 (XAF1), whose expression is indirectly related to heat shock transcription factor 1 (HSF1) activity. Indeed, the promoter region of the *XAF1* gene contains a heat shock element (HSE)-regulated transcription silencer, whose transcription negatively correlates with HSF1 levels (58). This evidence implicates a synergistic effect of two anti-apoptotic protein families in cytoprotection: the heat shock proteins (Hsps) and inhibitors of apoptosis. Actually, the role of Hsps as protective against the complete assembly of apoptotic machinery is well established (10, 43, 45). The importance of the Hsp system against oxida-

tive stress-induced apoptosis (23, 60) and the role of specific reactive cysteines in its redox-sensitive modulation (8, 17) have been previously addressed. Until now, the major new findings concerning the involvement of the Hsp system in apoptosis come from the HSF1 knockout mice that show a reduction of downstream Hsp25, αB-crystallin, and Hsp70 (59). These alterations are associated with a glucose 6-phosphate dehydrogenase-dependent decrease of the GSH/GSSG ratio, followed by an enhancement of mitochondrial ROS-deriving oxidized proteins, among which adenine nucleotide translocase 1 (ANT1) seems to be particularly affected. Oxidative damage to ANT1 protein, a structural component of the mitochondrial permeability transition pore (MPTP), decreases its catalytic activity and increases MPTP opening, thus sensitizing cells to apoptosis (14, 59).

By contrast, a pro-apoptotic role for the Hsp system has been reported in spermatocytes from mice expressing a constitutively active form of HSF1 that undergo mitochondrial-dependent apoptosis, mimicking heat shock-induced death of spermatogenic cells (57). A strengthening role of heat shock has also been suggested to be operative in TRAIL-induced apoptosis of transformed T lymphocytes, although it does not seem to be ascribed to Hsp neosynthesis (39). However, besides these exceptions, the Hsp system is generally associated with resistance to cell death. This could represent a benefit, as reported for the role of HSF1 in cochlear protection, recovery, and/or repair following noise overstimulation (21), or a dangerous side effect, such as in cancer. In fact, elevation of Hsp levels is widespread in cancer and predicts a poor prognosis and resistance to therapy. Recently, it has been proposed that this resistance can be induced by the highly malignant factor heregulin β1 (HRG β1), which, upon binding to the cell surface c-erbB receptors, leads to the inhibition of the glycogen synthase kinase 3, the intracellular antagonist of HSF1 (34).

Especially for what intrinsic apoptotic pathway concerns, mitochondria are the cellular compartment at the level of which anti-apoptotic proteins have to prevalently block apoptogenic stimuli. From a *fairytale* point of view, the mitochondrion represents the castle in which two opposite factions, the guardians (anti-apoptotic) and the conquerors (pro-apoptotic)—most of which are even blood relatives (*i.e.*, the Bcl-2 superfamily members)–try to win the battle. The conquerors generally storm the castle by means of: (a) reaching onto the walls (membrane insertion), (b) blocking their enemies (protein/protein interaction), (c) opening the walls (pores and MPTP formation), and (d) chasing away and dispersing the inhabitants (cytochrome *c* and other factors release). Within this imaginary world, the oncoprotein B cell lymphoma-2 (Bcl-2) is certainly one of the bravest defenders of mitochondrial integrity and inducers of cell survival. In fact, a therapeutic use has been proposed for Bcl-2-deriving modified peptides to inhibit neural cell death (50). Besides its well-established role as anti-apoptotic protein, a huge body of evidence has also suggested an additional antioxidant function for Bcl-2 (47, 56). This supplementary feature, already proposed by Hockenbery and co-workers in 1993 (30) was based on the observation that Bcl-2 was located at the mitochondrion, the primary source of ROS. On the contrary, 2 years later Steinman found a significant pro-oxidant activity for Bcl-2 (51), evidence that was not adequately supported by the scientific community. Actually, the knowledge at that time was still anchored to the notion that oxyradicals should only act as damaging molecules, raising the question: "How can an anti-apoptotic protein be a pro-oxidant?" Today, this apparent contradiction is going to be solved, thus so pro- or anti-oxidant can be, occasionally, represented by the same species. In fact, it has been demonstrated that Bcl-2 overexpressing cells show a significant, but subpathological, enhancement of ROS output that, in turn, stimulates the antioxidant defense. This phenomenon is tightly related to the effect that Bcl-2 has on mitochondrial outer membrane composition and matrix volume (for a review, see Ref. 36). These features are also the result of the activity of other proteins, such as the mitochondrial ATP-sensitive or insensitive K^+ channels, and seem to be essential, mainly during ischemia/reperfusion, in controlling NADH and ATP levels, permeability transition, and cell death (18–20). Recently, further evidence in favor of the pivotal role that Bcl-2 plays in maintaining mitochondrial redox homeostasis has been proposed. Indeed Cu, Zn superoxide dismutase (SOD1), especially the mutated amyotrophic lateral sclerosis (ALS)-related isoforms, has been found to directly interact with Bcl-2 (44), thus decreasing its anti-apoptotic effect and stimulating neurodegenerative processes. In this context we demonstrated that the interaction Bcl-2/SOD1 is operative also under resting conditions; in particular, decrease of SOD1 by means of specific siRNA, was associated with a rapid carbonylation and degradation of Bcl-2, which represents the causative event of a decrease in ATP content and intermembrane space potential ($\Delta\Psi$) (7).

REDOX STRESS-SELECTIVE CELLULAR RESPONSES

Cellular responses to redox stimuli seem to be able to discriminate between different ROS species, as observed for H_2O_2 *versus* superoxide anion ($O_2^{\bullet -}$) in regulating cell viability. Clement and co-workers previously suggested that the former represents an efficient pro-apoptotic molecule, whilst the latter behaves, at low concentrations, as a pro-survival factor (15). The molecular mechanisms underlying this phenomenon seems to be related to the opposite response of Na^+/H^+ exchanger 1 (NHE-1) to $O_2^{\bullet -}$ or H_2O_2, which positive or negative regulate its transcription, respectively (6). The results obtained indicate that *NHE-1* is a redox regulated gene that provides a novel intracellular target for the redox control of cell death sensitivity. Moreover, the authors speculate that, although H_2O_2 and $O_2^{\bullet -}$ might be assumed to virtually represent the same molecule under physiological pH, being H_2O_2 the product of very fast spontaneous (10^5 $mol^{-1}sec^{-1}$) or SOD-mediated (10^9 $mol^{-1}sec^{-1}$) one-electron $O_2^{\bullet -}$ reduction, they mediate unique (and perhaps opposite) intracellular responses. Regarding $O_2^{\bullet -}$, one of the most important characteristics involves its *intimate* relationship with nitric oxide (NO) (11). The encounter between these molecules can very rapidly (10^8 $mol^{-1}sec^{-1}$) lead to the formation of the damaging

and highly reactive nitrogen specie (RNS) peroxynitrite ($ONOO^-$). However, this interaction is regulated by the relative concentrations and by the activity of SOD and NO synthase (NOS), which modulate their abundance. It has been recently demonstrated that ROS and NO mutually, and in a concentration-dependent fashion, contribute to the modulation of the stabilized form of hypoxia-inducible factor-1α (HIF-1α) (12, 35), the inducible subunit of HIF-1, factor that controls several pathophysiological conditions (*i.e.*, chemoresistance; for review, see Ref. 61), and several key aspects of inflammation (16). The capability to act differently in a concentration-dependent manner seems to be a NO prerogative. In fact, especially for what cell death response concerns, the examples are numerous in which NO can induce (*e.g.*, by activation of p53/p21 system), or inhibit (*e.g.*, by *S*-nitrosylation of caspases) apoptosis, in relation to the amount released within the cell.

The *Janus* face molecule of NO allows considering new aspects in the redox-signaling network of apoptosis. Therefore, the initial idea of response selectivity would be generalized so that not only *different ROS species*, but also *different redox stresses* can specifically activate diverse cell responses. Whether verified, this hypothesis could be of great importance, since on the basis of the site of origin of ROS, their molecular characteristics, and the nature of resulting oxidative stress, it should be conceivable to determine the degree and the direction of cell response. In this context, recent evidence from our laboratory let us presume a preferential activation of specific members of MAP kinase family in response to oxidative stresses of different origin: disulfides *versus* ROS. This arises from the different responses observed in promonocytic U937 and neuroblastoma SH-SY5Y cells exogenously treated with GSSG, a nonpermeable pro-oxidant compound. Whereas the first histotype undergoes apoptosis by means of disulfide-dependent phospho-activation of Trx/ASK1/$p38^{MAPK}$ apoptotic pathway (26), neuroblastoma cells are completely resistant. However, these cells can be committed to apoptosis upon GSSG treatment under conditions that allow a ROS-dependent JNK/c-Jun mediated phosphorylative cascade (22). The preferential activation of JNK in neuroblastoma cell model has been also confirmed by the finding that the dissociation of JNK/glutathione-*S*-transferase (GST) heterocomplex is involved in cell death response upon ROS-dependent oxidative stimuli (25). The histotype selective activation could be more intriguing, since unpublished results from our laboratory give evidence that other cell lines, which have been demonstrated to be resistant to ROS-mediated activation of GST/JNK/c-Jun pathway (24), show a high sensitivity towards GSH-depleting agents (*i.e.*, diamide).

Overall reported data point out that the field of redox signaling, so far focused on ROS as second messengers, will be one of the aspects to be more deeply investigated in the near future, as some still nonanswered questions need to be clarified. In particular: where, by which, and how much ROS are produced upon specific apoptotic stimuli are the arguments that will require more experimental effort. Each novel piece of ROS-mediated signaling network, put in the right place, should help in explaining the different cellular responses, especially for a use of redox-active tools in the therapy of several pathologies where oxidative stress seems to have a key role, such as neurodegeneration and cancer.

ABBREVIATIONS

ALS, amyotrophic lateral sclerosis; ANT1, adenine nucleotide translocase 1; ASK1, apoptosis signal-regulating kinase 1; Bcl-2, B cell lymphoma-2; ERK1/2, extracellular signal-regulated kinase 1/2; GSH, reduced glutathione; GSSG, glutathione disulfide; GST, glutathione-*S*-transferase; HIF-1, hypoxia-inducible factor-1; HRG β1, heregulin β1; HSE, heat shock element; HSF1, heat shock transcription factor 1; Hsps, heat shock proteins; IKK, IκB kinase; IRF3, interferon regulatory factor 3; LPS, lipopolysaccharides; MAP kinase, mitogen activated protein kinase; MPTP, mitochondrial permeability transition pore; NF-κB, nuclear factor κB; NHE-1, Na^+/H^+ exchanger 1; NO, nitric oxide; NOX4, NADPH-dependent oxidase 4; NQO1, NAD(P)H:quinone oxidoreductase 1; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; TLR4, Toll-like receptor 4; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; Trx, thioredoxin; XAF1, XIAP-associated factor 1; XIAP, X-linked inhibitor of apoptosis.

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