Oxidative stress-induced apoptosis – An overview

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Apoptosis is a form of regulated cell death that has attracted a lot of interest in the recent years. Exposure of various cell types to oxidative stress-causing agents can directly induce apoptosis which can be blocked by a wide range of antioxidants. It is now believed that oxidants may be essential biochemical intermediates in the progression of many forms of apoptosis induced by different stimuli. Oxidative stress-induced apoptosis is an outcome of alterations of various metabolic pathways resulting in the loss of ATP and calcium homeostasis, DNA damage, mitochondrial permeability transition, and structural as well as functional modification of certain proteins. This form of apoptosis also involves the interplay of certain genes such as *Bcl-2*, *p53* and *c-myc* that may regulate the process either positively or negatively. In this review, an attempt has been made to elucidate, in brief, the role of these factors in oxidative stress-induced apoptosis.

APOPTOSIS is a type of cell death in which an individual cell undergoes an internally-controlled transition from an intact metabolically active state into a number of shrunken remanants retaining their membrane bound integrity[1,2]. This form of cell death is involved in many physiological and pathological processes. This is an essential process in controlling tissue homeostasis in multicellular organisms like nematodes, amphibians and mammals and is a way by which damaged, infected or neoplastic cells are continually eliminated without inducing any inflammatory response[3]. Apoptosis is sometimes referred to as programmed cell death (PCD) because it is an integral part of the developmental programme and is frequently the end result of temporal course of cellular events.

Apoptosis can be induced by a variety of stimuli such as ionizing radiations, gluco-corticoids, chemotherapeutic agents, lymphokines deprivation and various oxidants[4–9]. Although the stimuli which induce apoptosis vary markedly, the morphological features of the process are however conserved in different cell types[2].

Oxidative stress and apoptosis

Many of the chemical and physical stimuli capable of inducing apoptosis are known to evoke oxidative stress by increasing the steady state concentration of reactive oxygen species (ROS) (Figure 1). The formation of ROS within the cell occurs (i) as a consequence of mitochondrial consumption of oxygen[10]; (ii) via hydrogen peroxide production in peroxisomes; (iii) in the 'respiratory burst' during activation of phagocytes; and, (iv) during induction of cytochrome P450 enzymes[11]. It is interesting to know that ROS, which are highly reactive and nonspecific molecules can induce and mediate the well co-ordinated and controlled changes that occur during apoptosis[12,13]. For example, low concentration (5–40 μM) of H_2O_2 and nitric oxide (NO) induce apoptosis in different cell systems[14,15].

Some of the agents which cause apoptosis may not be free radicals but induce ROS production and/or decrease the reduction capacity of the cell. Depletion of various intracellular antioxidants, like glutathione (GSH) by using buthionine sulfoxamine, renders cells more susceptible to oxidative stress-induced apoptosis. Alternatively, antioxidants that scavange peroxides and other free radicals, e.g. *N*-acetylcysteine (NAC), thioredoxin, intracellular thiol reductase, glutathione peroxidase, endogenous thiols like dihydrolipoic acid, etc. rescue cells from apoptosis induced by different stimuli[4–6, 16–21].

Tumour necrosis factor (TNF) required for host defence against intracellular bacterial infections, exerts its cytotoxic effects via generation of intracellular ROS which induce apoptosis[22–26]. Antineoplastic agents like doxorubicin, cisplatin and ether-linked lipids also induce both oxidative damage and apoptosis in tumour cells[27]. Ultraviolet and ionizing radiation also induce apoptosis in different cell systems probably by generating ROS[7–9]. Glucocorticoid-induced apoptosis in thymocytes also shows an increased level of oxidation both in pre-apoptotic and apoptotic cells[28,29]. HIV-infected T cells are also extremely susceptible to oxidative stress-induced apoptosis due to an HIV-associated decrease in manganese superoxide dismutase (Mn-SOD) and catalase activity[30–32]. Apoptosis induced by HIV1, TNF and glucocorticoids can be blocked by exogenous antioxidants[33,34]. These findings suggest that oxidation plays a role in the progression of apoptosis, though neither the identity nor the site of generation or molecular targets of the putative oxidant species have been identified so far.

Studies have shown that ROS are implicated in apoptosis and may provide effector mechanism for the final

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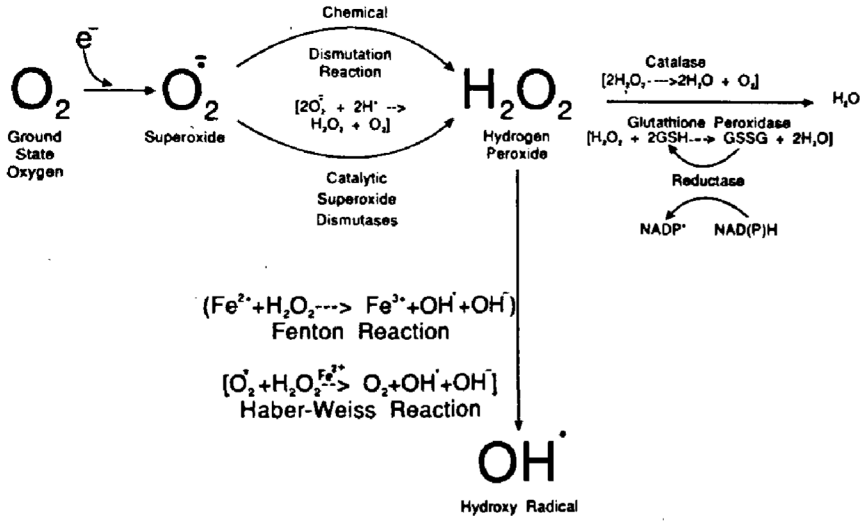

Figure 1. Generation of ROS in cells. The major forms of ROS and their metabolism in cells.

common pathway of apoptotic cell death[4,5]. However, overproduction or decreased elimination of O_2^- has recently been seen to provide tumour cells/activated cells with a survival advantage over normal counterparts/quiescent cells rather than causing apoptosis of the cells[35]. It has been observed that O_2^-, the primary ROS generated by normal cellular metabolism, can abrogate FAS-mediated apoptosis in cells which are constitutively sensitive to FAS[36]. The mechanism of O_2^--induced resistance to FAS signals is still not clear. But it seems that ROS may also have the beneficial effects, probably at concentrations which do not overwhelm the endogenous cellular protective mechanisms against ROS-induced damage.

Apoptosis and gene activity

One of the most intriguing concepts generated by the study of apoptosis is that this form of cell death is a genetically mediated suicide process. It is generally regulated by the induced synthesis of new gene products[37]. However, apoptosis also occurs independent of the synthesis of new gene products, e.g. in case of HL-60 human leukemic cell line and TNF-susceptible cell lines, where cells undergo increased apoptosis when macromolecular synthesis is inhibited by actinomycin D or cycloheximide[38,39]. The genetics of apoptotic cell death are best worked out in the nematode *C. elegans*, where the most important molecular regulators of apoptosis are the genes *Ced-3*, *Ced-4* and *Ced 9* (ref. 40). *Ced-3* and *Ced-4* are required for apoptosis to occur while *Ced-9* functions as its suppressor[40–42]. Mammalian homologs of these *Ceds* have been discovered. *Ced-9* is most homologous to mammalian proto-oncogene *Bcl-2* and also shows homology to the other members of *Bcl-2* family[43]. *Ced-3* encoding a cysteine protease with aspartic acid specificity (caspases) is the archetype of a family of caspases that are key effector proteins of apoptosis in mammalian cells[44]. *A*poptosis *a*ctivating *f*actor-1 (Apaf-1) has recently been reported to be a mammalian homolog of nematode protein *Ced-4* (refs 45, 46). Genetic studies have revealed that *Ced-4* is required for *Ced-3* function whereas *Ced-9* regulates apoptosis by preventing activation of caspase encoded by *Ced-3* (refs 47, 48). Another important molecular regulator of apoptosis in mammalian cells is the *c-myc* oncogene involved in coupling the cell response to cell proliferation[49]. Given that proto-oncogenes control the induction of apoptosis, it is not surprising that tumour suppressor genes such as *p53* are also involved. Overexpression of *p53* gene generally induces rapid cell death by apoptosis[50]. Interaction between growth promoting proto-oncogenes such as *c-myc* and *Bcl-2* and *p53* is known to regulate apoptosis[51]. For example, high concentration of *Bcl-2* can protect various cells from apoptosis induced by *c-myc*[51] and the co-expression of *c-myc* and *Bcl-2* genes can overcome *p53* induced apoptosis and cell cycle arrest[52]. Both the proto-oncogenes and the tumour supressor genes play a putative role in free radicals/oxidants induced cell death as is discussed in the following.

Oxidative stress, Bcl-2 and apoptosis

Bcl-2 was initially described as the oncogene that was present in the immunoglobulin locus as a result of translocation [*t*(14:18)] found in human B cell leukemias and lymphomas[53–55]. In 1988, it was shown that introduction of this gene into the interleukin-3-dependent

myeloid and lymphoid cell lines promoted survival of these cells after withdrawal of IL-3 (ref. 56). Subsequently *Bcl-2* was shown to specifically inhibit apoptosis[57]. *Bcl-2* is the founding member of a multigene family that consists of proteins homologous to *Bcl-2* encoded by mammals down through evolution to the nematodes. *Bcl-2* family members come in two functional categories – (1) inhibitors of apoptosis like *Bcl-2*, *Bcl-xL*, *Bcl-w*, *Mcl-1*, etc.[58–60] and (2) promoters of apoptosis such as *Bcl-xS*, *Bax*, *Bad*, *Bak*, etc.[59,61–63]. The ratio of the level of death inhibiting to death promoting *Bcl-2* family members determines the cell viability. One mode of control of apoptosis by *Bcl-2* takes place at the level of protein–protein interactions through which the *Bcl-2* family members form both the homo and heterodimers[61]. For example, *Bax* is a *Bcl-2* homolog that possesses intrinsic death activity[61] and its dimerization with *Bcl-2* influences a cell's response to apoptotic stimuli[64].

Bcl-2 can block apoptosis induced by a variety of stimuli such as nerve growth factor withdrawal[65,66], irradiation[67], cancer chemotherapeutic agents[68], glucocorticoids[14] and *c-myc*[69]. This suggests a central role for *Bcl-2* in the regulation of cell death from diverse signalling mechanisms. Studies have shown that *Bcl-2* may suppress apoptosis by functioning as an antioxidant[19,70,71]. Because apoptosis induced by stimuli which cause oxidative stress (ionizing radiation, heat shock, TNF, inhibition of glutathione (GSH) synthesis or generation of superoxides) is blocked by *Bcl-2*[14,57,72–76]. It is observed that (i) increased synthesis of intracellular *Bcl-2* blocks the increase in ROS associated with apoptosis[70]. (ii) *Bcl-2* inhibits ROS-induced apoptosis[19] and (iii) various antioxidants like NAC can substitute for *Bcl-2* expression[19].

Free radicals independent protection by *Bcl-2* was also observed, *Bcl-2* did not reduce the ROS production and yet it protected the cells from H_2O_2-triggered apoptosis[77]. *Bcl-xL*, a homolog of *Bcl-2*, also rescued B lymphocytes from oxidant-mediated death caused by diverse apoptotic stimuli without affecting the ROS formation[78]. *Bcl-2* could even inhibit hypoxia-induced apoptosis[79,80]. Whatever antioxidant or ROS inhibitory properties *Bcl-2* may have, these are not necessary for it to inhibit apoptosis.

The location of *Bcl-2* gene product primarily on the outer membrane of mitochondria raises the possibility of its relation to the function of mitochondria (a site of oxidative phosphorylation and adenosinetriphosphate (ATP) generation) which has been implicated in apoptosis[81–83]. Yang *et al.* observed that the overexpression of *Bcl-2* blocked the efflux of cytochrome c from the mitochondria, thus preventing apoptosis in human HL 60 cells[84]. The cytosolic preparation containing the cytochrome c also induces apoptosis by activating a cysteine protease CPP32 involved in this process[85]. It is now well known that *Bcl-2* inhibits cytochrome c translocation which blocks caspase (CPP32) activation and the subsequent apoptosis of the cell[84,86].

Recent studies have shown the role of *Bcl-2* in modulating the mitochondrial transmembrane potential ($\Delta\psi_m$). An intact $\Delta\psi_m$ is indispensable for the normal mitochondrial function – a prerequisite for the survival of a cell. Disruption of the intact $\Delta\psi_m$ causes mitochondrial permeability transition (PT) (a sudden increase in the permeability of the inner mitochondrial membrane to solutes $\leq$ 1500 Da such as protons, calcium, glutathione, etc.) which consequently evokes apoptosis[87–90].

Various oxidizing (H_2O_2, *t*-butylhydroperoxide and peroxynitrite) and redox generating agents (menadion and alloxan) modulate mitochondrial permeability transition[87,88,90]. *Bcl-2* overexpression in the mitochondrial membrane inhibits the permeability transition induced by a variety of these PT inducers[90] and prevents the disruption preceeding apoptosis induced by various oxidants[87–90]. *Bcl-2* while inhibiting PT prevents the release of small molecules, such as calcium from the mitochondrial matrix[91] and that of apoptosis inducing factor (AIF) from the intermembrane space which are well known inducers of apoptosis[90–92]. PT itself leads to the changes in the cytoplasmic redox state such that depletion of reduced glutathione and enhanced generation of ROS occurs. The depletion of reduced glutathione and production of ROS also invariably precede the mitochondrial permeability transition due to a fall in mitochondrial transmembrane potential thus leading to apoptosis of the cell[93]. *Bcl-2* can therefore, inhibit both the effects of ROS (via inhibition of ROS mediated PT) and the generation of ROS (which is a consequence of PT) (Figure 2).

Oxidative stress, p53 and apoptosis

Among the tumour suppressor genes, a lot is known about *p53*, a gene which is inactivated by mutation in the majority of human cancers[94]. *p53* is generally upregulated by stimuli which induce DNA damage and blocks the progression of the cell cycle from G_1 to S phase[94–96]. By this mechanism *p53* prevents the propagation of the genetic lesions in cellular progeny. In addition to G1 arrest, it is known that *p53* is also a mediator of apoptotic cell death both in malignant and normal cells of different lineages[97–101]. Whether the cell undergoes *p53*-mediated G1 arrest or apoptosis appears to depend on the cell type and the degree of DNA damage[102].

A role for *p53* in apotopsis induced by free radicals and oxidants has also been reported[103,104]. Nitric oxide (NO) generation in response to a cytokine-induced NO synthase or NO donors stimulate the *p53* expression prior to apoptosis[103]. Inhibitors of nitric oxide synthase,

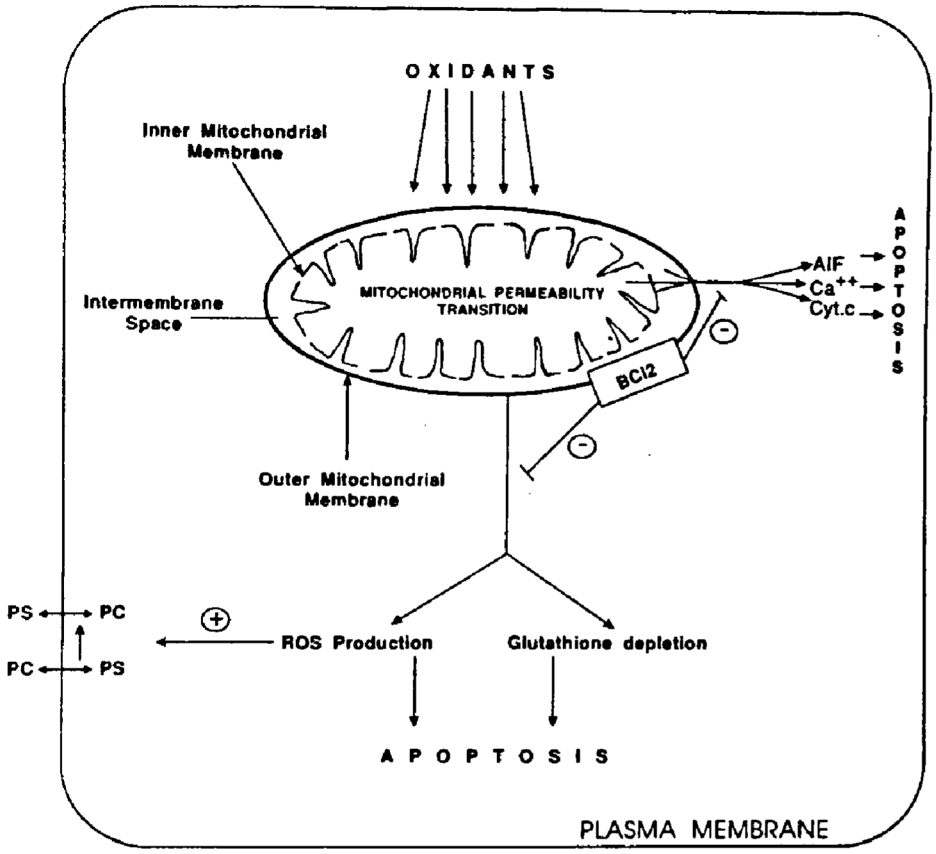

Figure 2. Oxidants, mitochondria and apoptosis: Oxidants induce mitochondrial permeability transition (PT). The *Bcl-2* oncoprotein regulates PT induction in response to oxidants. As a consequence of PT, mitochondria release apoptosis inducing factor, cytochrome C and calcium. In addition, PT causes the generation of reactive oxygen species, rapid expression of phosphatidylserine residues in the outer plasma membrane leaflet and depletion of reduced glutathione. These changes form part of the degradation phase of apoptosis beyond the point of no return. Interrupted inner mitochondrial membrane depicts the permeability transition induced by oxidants. (AIF: apoptosis inducing factor; Cyt C: cytochrome C; Ca^{++}: calcium; ROS: reactive oxygen species; PS: phoshatidylserine; PC: phoshatidylcholine.)

NG-monomethyl-L-arginine, prevent generation of NO as well as the corresponding *p53* expression and apoptosis[103]. ROS-mediated DNA damage also causes the accumulation of *p53* which is associated with apoptosis[104]. ROS not only induce DNA damage directly but also affect other targets within the cell, thereby inducing the apoptotic pathway independent of genomic DNA damage. It is, therefore, possible that *p53* may be indirectly increased by other factors induced by ROS, for example nuclear factor kB which is known to be induced via free radical reactions may activate *p53* as a transcription factor[105].

There are certain studies in which reactive oxygen species are shown to be the downstream mediators of *p53*-dependent apoptosis[106,107]. For example, smooth muscle cells sensitive to *p53*-mediated apoptosis, produced ROS concommitantly with *p53* expression whereas cells resistant to *p53* failed to produce ROS[106]. In sensitive cells both ROS production and apoptosis were inhibited by antioxidant treatment[106]. Decreasing the intrinsic oxidative level in myeloid leukemic cells by antioxidants such as butylated hydroxyamisole (BHA); cimetidine (CIM), *N*-butyl-L-phenyl nitrone (BPN) and *N*-acetyl cysteine (NAC) also protected cells from wild type *p53*-induced apoptosis whereas increasing the intrinsic oxidative level by adding H_2O_2 enhanced apoptosis[107]. Thus *p53* may regulate the intracellular redox state which in turn may induce apoptosis by activation of certain targets downstream from *p53*. Evidences now indicate that *p53* is one component of a signal pathway involving activation of multiple downstream effector genes such as $p21^{WAF1/CIP1}$, *GADD45*, *MDM2* involved in apoptosis[108,109]. The production of free radicals and modulation of intrinsic oxidative levels may trigger these secondary components of apoptotic cascade resulting in apoptosis. In fact, it is seen that in the case of proliferating human fibroblasts, ROS induce apoptosis by increasing both the p53 and $p21^{WAF1/CIP1}$ protein levels[110].

Oxidative stress, c-myc and apoptosis

The *c-myc* proto-oncogene is another important regulator of apoptosis and is involved in coupling the response to proliferation. The first evidence that *c-myc* expression can promote apoptosis came from the experiments of Cleveland *et al.*[111] who observed that overexpression of *c-myc* in an interleukin (IL)-3 dependent cell line accelerated apoptosis upon growth factor withdrawal. Additional evidences came from the study of Bissonnette *et al.*[112] who found that antisense oligonucleotides to *c-myc* blocked T cell receptor-mediated apoptosis in T cell hybridomas.

c-myc induced apoptosis is inhibited by various antioxidants[4,19,70]. Simultaneous overexpression of *Bcl-2* proto-oncogene also abrogates the capacity of increased *c-myc* expression to induce apoptosis, a fact that may be of importance in the synergistic involvement of these two genes in oncogenesis[111–114]. Ornithine decarboxylase (ODC) has been shown to be an effector of *c-myc*-induced apoptosis[115]. It is believed that *c-myc* induced ODC activity results in increased rates of polyamine synthesis and catabolism, thereby generating potentially lethal excess of ROS which induce apoptosis subsequently. It shows that ROS or some products of ROS are the common mediators of apoptosis in many systems.

Potential mechanisms of oxidative stress-mediated apoptosis

DNA damage

The action of ROS on nuclear material can result in base modifications, base free sites, single and double strand breaks and crosslinks[116]. Production of ROS from (1) macrophages and neutrophills, (2) by cell respiration and (3) the loss of normal cell peroxidases and the subsequent conversion of H_2O_2 to the more oxidatively reactive OH· (by metal ions, especially iron and copper that are bound at or close to DNA) can result in a high degree of direct oxidation of nucleic acids which can initiate an apoptotic response[117–119]. Chromatin, especially histones, has also been shown to be the primary target of ROS. Its fragmentation can occur by the activation of endonucleases either directly by ROS or indirectly by the oxidative stress-induced Ca^{++} excess[120].

Membrane lipid peroxidation and the loss of calcium homeostasis

Though the role of lipid peroxidation in apoptosis remains largely unexplored, the potential target for oxidative damage within the cell, i.e. membrane phospholipids, lipid-derived mediators such as ceramide and fatty acids hydroperoxides, have however been implicated as regulators of apoptosis[121,122]. It has been observed that cells undergoing apoptosis in response to a variety of stimuli rapidly express phosphatidylserine (PS) on their outer surface. Phosphatidylserine is a phospholipid located on the inner leaflet of the plasma membrane and its expression on outer surface of the plasma membrane helps in their recognition and subsequent phagocytosis limiting tissue inflammation[123,124]. The externalization of the PS to cell surface has been shown to be mediated by its peroxidation which precedes the externalization and is inhibited by *Bcl-2* (ref. 125). Several intracellular enzymes, such as protein kinase C & raf 1 kinase, with signal transducing properties are modulated on binding PS[126,127]. Therefore, PS externalization is believed to play a key role in signalling apoptosis after oxidant stress.

Additionally, the oxidation of systemic and membrane lipids and the activation of the phospholipases by oxygen species increase the amount of arachidonic acids (AA) metabolites, peroxides, aldehydes and oxysterols. These processes promote increased production of free radicals, peroxidation of membranes, membrane blebbings Ca^{2+} influx resulting in loss of calcium homeostasis which is a critical factor for cell survival[32]. The oxidized products of the lipids induce apoptosis as well as alter cytosolic calcium homeostasis[32,128,129]. It is possible that modulation of calcium homeostasis by the oxidized lipids leads to the activation of various enzyme systems such as protein kinase C, endonucleases, proteases and phospholipases which in turn are responsible for mediating cellular demise in apoptosis.

Alterations of metabolic pathways and ATP loss

ROS-mediated DNA damage elicits the activation of poly-ADP-ribose transferase, responsible for polymerization of ADP-ribose to proteins, which results in a rapid depletion of cellular NAD/NADH pools and the collapse of ATP stores leading to apoptosis[4]. The oxidative stress-induced inhibition of glyceraldehyde 3-phosphate dehydrogenase, an important NADH generating enzyme, due to the oxidation of its thiol portion (Cys 149) also results in a significant loss of ATP which consequently evokes apoptosis[32,130].

Alteration of certain proteins and transcription factors

Oxidation of the critical SH (sulfhydrals) present in the calcium (Ca^{2+}) transport systems, located in the endoplasmic reticulum, mitochondria and plasma membrane, may promote increase of calcium in the cytosol. This may subsequently trigger various components of the

apoptotic effector pathways[29]. Oxidation of certain intracellular proteins may modify either their function or their ability to be recognized by other proteins. For example, oxidative damage can increase the susceptibility of some proteins to degradation by the nonlysosomal proteases[131]. Protease inhibitors are known to block thymocyte apoptosis and it is possible that these might be protecting the same intracellular targets as antioxidants from degradation[132].

Oxidation of caspases, the cysteine proteases involved in apoptosis, also affects their activity. Depending upon the degree of initial oxidative stress, the caspases are either activated and the cells die by apoptosis, or they remain inactive and necrosis occurs[133]. The observed resistance of certain cell lines to Fas induced apoptosis may also be because of the oxidative inactivation of caspases.

Certain transcription factors are also regulated by the redox state of the cells. Transcription factors (c-fos and c-jun (AP-1), p53, NF-kB, c-myc, etc.) have critical cysteine residues involved in DNA binding. The oxidation of cysteine residues causes a large decrease in their efficiency for DNA binding[134,135]. Thioredoxin (a dithiol reducing enzyme) and ref-1 (a DNA repair enzyme) known to regulate the redox state of both the AP-1 and NF-κB are themselves also redox regulated[136,137].

The intracellular oxidative events may also affect certain transcription factors indirectly. For example, DNA-binding activity of nuclear factor κB(NFκB) is indirectly activated by oxidative events (via enhanced proteolysis of its inhibitory factor iκB), initiating transcription of NFκB responsive genes[138,139]. Oxidation, therefore, has the potential to alter the phenotype of a cell via changing gene transcription. And, in some situations this may provide an entrance to apoptosis.

Summary

This article summarizes the recent findings that describe both the role and mechanisms of oxidative stress-induced apoptosis. In brief, intracellular oxidation/oxidative state may play a central role in apoptotic cell death than has hitherto been assumed. It is also clear that not only can the oxidative stress induced by different stimuli induce apoptosis but various antioxidants also protect the cell against apoptosis even when induced by stimuli that do not exert a direct oxidant effect. Oxidative stress-induced apoptosis seems to be the product of multiple pathways resulting in the oxidation of membranes, loss of Ca^{2+} homeostasis, diffuse activation of enzyme systems (including activation of endonucleases), disruption of metabolic processes (including the changes in the mitochondrial transmembrane potential), ATP loss, and, alterations in certain proteins and transcription factors. A growing body of work has also suggested that oxidative stress-induced apoptosis involves alterations in the expression of the tumour supressor (*p53*) and the promoter genes (*Bcl-2* and *c-myc*). Thus, apoptosis induced by oxidative stress is also subject to many checks and balances between different genes. It is attractive to consider that further insight into the regulation of oxidative stress-induced cell death might lead to new therapies for oxidative stress-induced neurodegerative and heart diseases, carcinogenesis, ageing, etc. Nevertheless, much of the details of oxidative stress-induced cell death pathways are still to be resolved.

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