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Glutathione depletion-induced chromosomal DNA fragmentation associated with apoptosis and necrosis

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Abstract

Chromosomal DNA and mitochondrial dysfunctions play a role on mammalian cell death induced by oxidative stress. The major biochemical dysfunction of chromosome is the presence of an ordered cleavage of the DNA backborn, which is separated and visualized as an electrophoretic pattern of fragments. Oxidative stress provides chromatin dysfunction such as single strand and double strand DNA fragmentation leading to cell death. More than 1 Mb of giant DNA, 200-800 kb or 50-300 kb high molecular weight (HMW) DNA and internucleosomal DNA fragments are produced during apoptosis or necrosis induced by oxidative stress such as glutathione (GSH) depletion in several types of mammalian cells. Reactive oxygen species (ROS)-mediated DNA fragmentation is enhanced by polyunsaturated fatty acids including arachidonic acid or their hydroperoxides, leading to necrosis. Mitochondrial dysfunction on decrease of trans membrane potential, accumulation of ROS, membrane permeability transition and release of apoptotic factors during apoptosis or necrosis has been implicated. This review refers to the correlation of chromosomal DNA fragmentation and apoptosis or necrosis induced by GSH depletion, and the possible mechanisms of oxidative stress-induced cell death.

Keywords: apoptosis - giant DNA fragmentation - GSH depletion - lipid peroxidation - mitochondria dysfunction - necrosis - oxidative stress

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Introduction

Although classification of cell death has proven difficult, based on the morphology of dying cells and on the DNA fragmentation or damages, two distinct patterns of cell death have been identified. These have been termed necrosis and apoptosis [1]. Mammalian cell death can be induced through chromosomal DNA damage by oxidative stresses such as ionizing and ultraviolet (UV) irradiation, anticancer drugs and various DNA adduct inducing substances. Under oxidative stress, reactive oxygen species (ROS) such as hydroxyl radicals (·OH), superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2) have been shown to damage chromosomal DNA and other cellular components, resulting in DNA degradation, protein denaturation, and lipid peroxidation. However, the mechanisms behind these cellular effects are rather complex, and are not yet fully understood. Some agents producing ROS induce cell death including apoptosis, providing lipid peroxidation and DNA damage [2]. However, the implications of lipid peroxidation for ROS-induced DNA damage remains to be elucidated. Furthermore, it is not clear whether ROS are a part of the signal transduction cascade triggered by various inducers for apoptosis, or whether they are generated in a parallel pathway that can independently trigger apoptosis. It is important therefore to distinguish between ROS molecules involved in such signaling pathways, and those that mediate general cellular damage including giant DNA and high molecular weight DNA fragmentation. Glutathione (GSH), an important soluble antioxidant in the brain, detoxifies H_2O_2 and lipid hydroperoxides. It has been suggested that a GSH loss may be an early event in neuronal diseases. Apoptosis is characterized by cell shrinkage, chromatin condensation, some caspase activations and fragmentation of DNA at internucleosomal linker sites [3]. Necrosis is an uncontrolled event resulting from loss of homeostasis and the cell contents are dispersed, which may then have adverse effects on neighboring tissue [4].

Our aim is to review chromosomal DNA fragmentations such as giant DNA, high molecular weight (HMW) DNA, and internucleosomal DNA fragmentations; and to reflect upon their significance in apoptosis or necrosis induced by oxidative stress under GSH depletion.

Patterns of chromosomal DNA fragmentation

Giant DNA fragment cleavage

Electrophoretic techniques, particularly pulsed-field gel electrophoresis, have been extensively applied and have broad application in biochemistry and genetics due to their sensitivity for the analysis of DNA molecules with length up to 10 Mbp [5, 6]. Pulsed-field gel electrophoresis has been used to analyze the precise molecular nature of DNA fragments produced by oxidative stress in cultured animal cells [2, 7, 8]. A more than 1 Mbp in size chromosomal DNA fragment is classified as a giant DNA fragment [9]. DNA degradation accompanied with DNA fragmentation producing 1-2 Mbp and 100-800 kbp DNA fragments were observed during cell death in T-24 human bladder cells treated with agents that produce ROS [2, 9], or under GSH depletion in rat glioma cells [7]. Chromosomal DNA fragments of 100-800 kbp and 50-300 kbp sizes are called high molecular weight (HMW) DNA fragments. The 1-2 Mbp giant DNA and 100-800 kbp NMW fragmentations that may represent features of high-order chromatin structures such as minibands and loops of DNA [10], lead to apoptosis as ascertained by internucleosomal DNA fragmentation in C6 cells [11]. However, little is known about the mechanism of giant DNA and HMW DNA fragmentation during apoptosis induced by ROS or other triggers.

Large DNA and internucleosomal DNA fragment cleavage

Wyllie [12] has popularized the notion that DNA fragmentation is a component of apoptosis. Studies using glucocorticoids and rat thymocytes as a model system showed that DNA was fragmented into 180-200 bp prior to cell death. This form of DNA degradation has been very widely observed in apoptosis, although exceptions do exist. One of the hallmarks of apoptosis is the digestion of genomic DNA by an endonuclease, generating a ladder of small fragments of double-stranded DNA. DNA fragmentation induced during apoptosis is not due

to a double-strand cutting enzyme as previously postulated, but rather is the result of single-strand breaks. This ensures the dissociation of the DNA molecule at sites where cuts within close proximity are found [13].

Chromosomal DNA fragmentation in apoptosis and necrosis

Apoptosis is characterized by chromatin condensation, activation of some caspases and fragmentation of DNA at internucleosomal linker sites giving rise to discrete bands of multiples of 180-200 base pairs [3]. In contrast, necrosis is a passive process, typified by cell and organelle swelling with spillage of the intracellular contents into the extra cellular milieu. Necrosis is an uncontrolled event resulting from the loss of homeostasis and may then have adverse effects on neighboring tissues [4].

In apoptosis, there is a two-step process of DNA fragmentation: DNA is first cleaved into large fragments of 50-300 kb and these are subsequently cleaved into smaller oligonucleosomes in some, but not all cells. Significantly, only the first stage is considered essential for cell death since some cells, for example human MCF7 breast carcinoma and human NT2 neuronal cells, do not show this behavior but still display normal nuclear morphological apoptotic changes [14]. Three types of DNA fragmentation occurring during apoptosis can thus be distinguished. Recently, other types of DNA fragmentation have been reported during apoptosis, in the presence or absence of the characteristic internucleosomal DNA cleavage (ladder-like) pattern [15]. Internucleosomal DNA is cleaved with fragmentation into large 50-300 kbp lengths and single-strand cleavage events. These enzymatic events encompass a vast array of chromosomal degradation states in cells with the universal consequence being cell death [16].

Some inducers of apoptosis have provided formations of 50-300 kbp HMW DNA fragments prior to internucleosomal DNA fragmentation in apoptotic MCF-7 cells induced by etoposide [14], and in mouse L-929 cells induced by tumor necrosis factor (TNF-α) [17]. These DNA fragment formations have been observed in several human epithelial cells induced by serum deprivation [18], and in HeLa nuclei treated with apoptosis-inducing factor (AIF) [15]. AIF was shown to induce nuclear condensation and large-scale DNA fragmentation to ~50 kb fragments. However, little has been reported about the involvement of not only 1-2 Mbp giant DNA fragmentation but also HMW DNA fragmentation to 100-800 kbp and 50-300 kbp fragments, and their significance or roles in apoptosis [19]. In some cases of apoptosis, ROS may be involved, not only as inducers of DNA damage but also as specific second messengers in the signal transduction pathway; whereas in others they may be side effects of either an experimental system or changes in the cellular redox status as a result of ROS-independent apoptosis signaling pathways [20]. Therefore, it is still unclear whether endogenous ROS are really involved in DNA degradation leading to apoptosis.

GSH depletion induces giant DNA fragmentation through ROS production

Oxidative stress is associated with a number of neurodegenerative diseases and has been found to induce apoptosis in neuronal cell lines [21] and cultured primary neurons [22] and it contributes to neuronal cell damage that results from various ischemic insults [23]. When C6 rat glioma cells were treated with glutamate, intracellular glutathione (GSH) was reduced to approximately one seventh of the initial level, and the reduction of GSH induced cytolysis was accompanied by apoptosis. Although the source was obscure, extracellular hydrogen peroxide was released without decaying and accumulated in C6 cells under glutamate-induced GSH depletion [7]. Hydrogen peroxide, which is continually generated within cells as a result of metabolic pathways such as xanthine oxidase, NADPH oxidase and monoamine oxidase (MAO), is a major candidate. In nerve cells, hydrogen peroxide production by the metabolism of catecholamines and indoleamines by MAO have been considered [24]. An enzyme distinct from MAO is involved in monoamine metabolism and demonstrates a relationship between glutamate toxicity and monoamine metabolism [25]. MAO catabolizes intraneuronal dopamine, which is present in high milimolar concentrations within the neuron, yield-

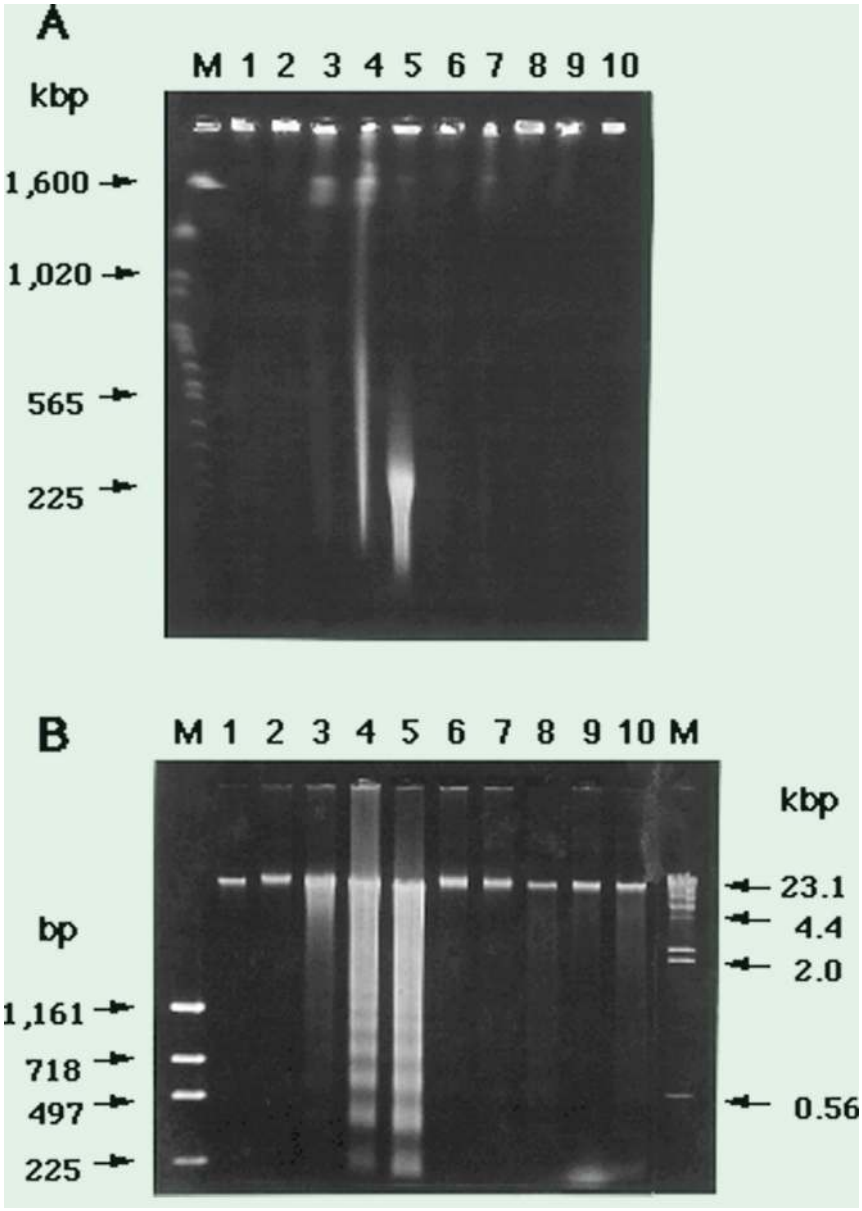

Fig. 1 Pulsed-field gel and agarose gel electrophoresis analysis of chromosomal DNA of C6 rat glioma cells treated with glutamate. A. Pulsed-field gel electrophoresis analysis of C6 rat glioma cells treated with glutamate in the absence (*lane 1-5*) or presence (*lane 6-10*) of antioxidants or metal chelator for the indicated times. *Lane 1*, control (glutamate free) for 6 h; *lane 2*, glutamate for 6 h; *lane 3*, glutamate for 9 h; *lane 4*, glutamate for 12 h; *lane 5*, glutamate for 24 h; *lane 6*, glutamate + N-acetyl cysteine for 12 h; *lane 7*, glutamate + ascorbic acid for 12 h; *lane 8*, glutamate + catalase for 12 h; *lane 9*, glutamate +deferoxamine for 12 h and *lane 10*, glutamate + 5,5'-dimethyl-1-pyrroline-N-oxide for 12 h. **B.** Agarose gel electrophoresis analysis of low molecular weight DNA obtained from the C6 cells treated as shown in *A*. Lane condition is the same as that of *A*. Lane M in both *A* and *B* indicates size marker DNA. (Reproduced with permission of Elsevier Press from Higuchi and Matsukawa [7]).

ing hydrogen peroxide stoichiometrically. As shown in Fig. 1, 1-2 Mbp chromosomal giant DNA and 200-800 kbp HMW DNA fragments were observed during apoptotic cell death induced by GSH depletion [7]. In glial and neuronal cells, glutamate induces GSH depletion and consequently apoptosis through endogenously produced active oxygen species, and apoptosis is accompanied by 1-2 Mbp giant DNA fragmentation prior to internucleosomal DNA fragmentation (Fig. 2). Such apoptosis is also induced under GSH depletion induced by L-buthionine-(S, R)-sulfoximine (BSO), an inhibitor of γ-glutamyl cysteine synthetase [11]. Furthermore, approximately 2 Mbp giant DNA fragments were observed in the BSO-treated cells. Giant DNA fragmentation was followed by approximately 100-800 kbp and 50-300 kbp and then less than 100 kbp including internucleosomal DNA fragmentations (Fig.1 and 2)

Under GSH depletion, hydrogen peroxide endogenously produced by MAO enhances arachidonic acid release through cellular phospholipase A_2 activation in membranes [26] and furthermore, might be converted to hydroxyl (OH) radicals by metals such as iron and copper. Li *et al.* [27] have reported that 12-lipoxygenase was activated and played an important role in nerve cell death in HT-22 hippocampus-derived cells under GSH deple-

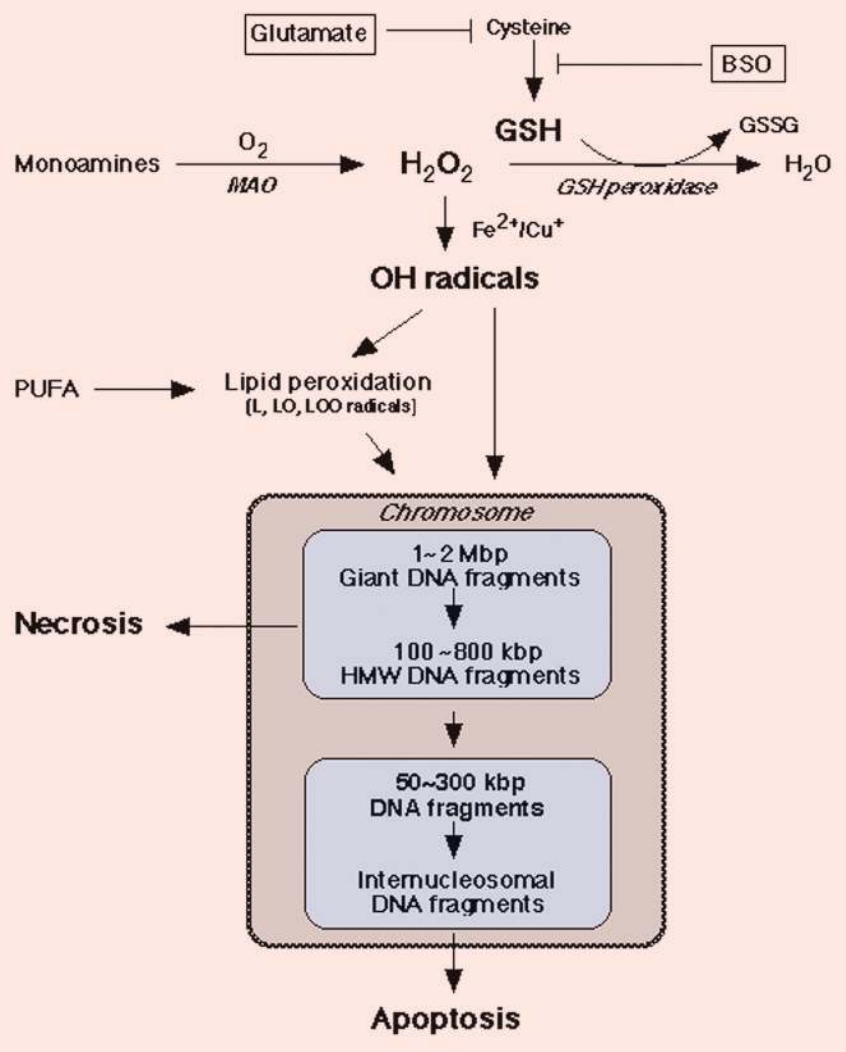

Fig. 2 Chromosomal DNA fragmentation under GSH depletion induced by glutamate in glial cells. Glutamate can inhibit cystine transport leading to both reductions of intracellular cysteine and GSH levels. Intracellular hydrogen peroxides produced in some pathways may be converted to OH radicals by metals such as ferrous iron or copper. The accumulated OH radicals might attack membrane phospholipid and chromosomal DNA, leading to lipid peroxidation and 8-OH-dG formation. 1-2 Mbp giant and 100-800 kbp high molecular weight DNA fragments formed in glutamate-treated C6 glioma cells are further cleaved into 50-300kbp and oligonucleotide fragments in apoptosis. These smaller DNA fragmentation induced by CAD/DFF40 is not occurred in necrosis. The excessive accumulation of lipid hydroperoxide under GSH depletion may cause cellular damages associated with necrosis.

tion. The expression and distribution of 12-lipoxygenase have been reported to be specific for cell types or tissues in animals [28]. Arachidonic acid induced an enhanced 1-2 Mbp giant DNA fragmentation and the disappearance of internucleosomal DNA fragmentation under GSH depletion in rat glioma cells [29]. A decrease of GSH triggered an activation of neuronal 12-lipoxygenase leading to the production of peroxides, the influx of Ca^{2+} and ultimately to cell death of H-22 hippocampus-derived cells [27]. Thus, exogenous arachidonic acid may potentiate cell death under conditions of GSH-depletion. Lipid metabolites, such as arachidonic acid-derived eicosanoids, may play a role in regulating cell survival [30].

The accumulated OH radicals might attack chromosomal DNA, leading to 8-hydroxy-2'-deoxyguanosine (8-OH-dG) formation. Beside OH radicals, lipid hydroperoxides or lipid alkoxyl radicals contributed 8-OH-dG formation under GSH depletion through oxidative modification of nuclear membrane integrity by lipid peroxidation [31].

A role of lipid peroxidatin in chromosomal DNA fragmentation

Poly unsaturated fatty acids (PUFAs) such as arachidonic acid, linolenic acid and linoleic acid markedly enhanced lipid hydroperoxide formation in C6 cells treated with glutamate or BSO. The 1-2 Mbp giant DNA fragmentation induced by BSO was enhanced by exogenous addition of PUFAs, depending on the species of PUFAs. The 100-800kbp HMW DNA fragments produced in both

glutamate- and BSO-treated C6 cells were also increased by the addition of PUFAs. Thus, the 1-2 Mbp giant DNA fragmentation is modulated by ROS or ROS-mediated lipid peroxidation.

The chromosomal DNA fragments with free 3'-OH termini were caused by the GSH depletion and enhanced by PUFAs in C6 glioma cells [29]. Thus, PUFAs could promote 1-2 Mbp giant DNA fragmentation with 3'-OH termini causing rapid necrotic cell death through lipid peroxidation. Lipid peroxidation producing lipid free radicals might proceed not only in plasma membranes but also in the nuclear membranes close to chromosomes, to the loss of membrane integrity in cell membranes consisting of phospholipids; and thereby may make circumstances suitable for oxygen radicals such as ·OH radicals to attack chromatin DNA.

Apoptosis or necrosis induced by GSH depletion

Although apoptosis and necrosis are mediated through distinct pathways, the same insult can lead to either apoptosis or necrosis depending on its intensity, the neuronal subpopulation involved, and the species and genotype of the organism involved. In many cell types both an apoptotic and a necrotic pathway are latently present. When upon stimulation, caspase-independent pathway is non-functional or blocked, an underlying slower pathway becomes in evidence, which leads to cell's demise by necrosis. In vitro, excitotoxic glutamate-induced cell death has been previously shown to cause a mixture of apoptosis and necrosis. Activation of glial cells induces release of arachidonic acid in astrocytes and microglia. Glial activation combined with GSH-depletion, which increases lipoxygenase activity, should result in excess free radical generation and create additional oxidative challenge to GSH depleted cells [27]. The cell death has been associated with caspase-3 activation in rat glioma cells [32]. Caspase-3 activation is an early biochemical marker of apoptosis in some types of cells induced by various triggers [33]. Ac-DEVD-CHO, a cell permeable specific inhibitor of caspase-3, inhibited BSO-induced apoptosis but did not in the presence of PUFA, in which the caspase-3 activity was reduced [32]. The caspase-3 activation may be involved in the mild cell death induced by BSO but not in the intense cell death in the presence of arachidonic acid under GSH depletion. CAD, caspase-activated DNase, has been shown to be involved in the internucleosomal DNA fragmentation during apoptosis induced by various apoptosis triggers [34]. However, it is not yet obvious which ROS or redox catastrophe induces caspase-3 activation and DNA ladder fragmentation at low levels or the depletion of GSH. Armstrong *et al*. [35] have reported activation of caspase-3 in cerebellar granule cells undergoing apoptosis but not necrosis.

PUFA such as arachidonic acid cause necrosis at high concentrations under GSH depletion, promoting 1-2 Mbp giant DNA fragmentation of chromatin and suppressing internucleosomal DNA fragmentation through lipid peroxidation inducing membrane integrity loss [29]. Therefore, it is likely that BSO or glutamate-induced GSH depletion initiates apoptosis through the caspase activation signaling system in C6 cells, while high concentrations of PUFA turns apoptosis into necrosis, estimated by alterations of not only caspase-3 activation but also internucleosomal DNA fragmentations [4, 34]. Activation of some phospholipases during necrosis, particularly cytosolic Ca^{2+}-dependent phospholipase A_2 (PLA_2), has been demonstrated [36]. PLA_2 is specific to substrate with arachidonic acid at the sn-2 position and its translocation to cell membranes. Lysophosphatidic acid, which is produced from phospholipids by removing arachidonic acid of PLA_2, also induced both apoptosis and necrosis in hippocampal neurons by unknown mechanisms [37]. Thus, it seems clear that not only lysophosphatidic acid but also other PUFAs cannot be ignored in the cell death associated with lipid peroxidation. The GSH depletion-induced oxidative cell death showed characteristics of both apoptosis and necrosis in HT-22 hippocampus-derived cells [38]. Excess accumulation of lipid hydroperoxides produced by lipid peroxidation under intracellular GSH depletion may promote cell death through necrosis. The change from apoptosis to necrosis accompanied with a reduction of internucleosomal DNA fragmentation may be dependent on the intensity of oxidative stress and lipid peroxidation.

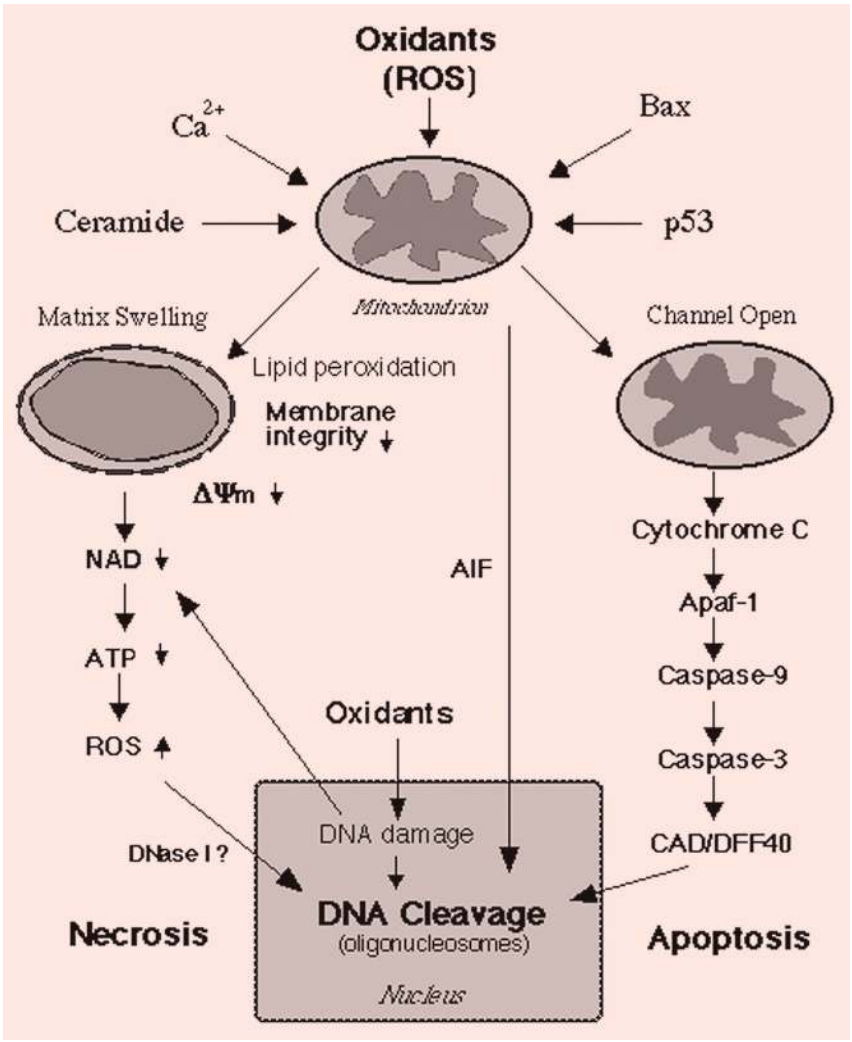

Fig. 3 Model for apoptosis and necrosis activation by mitochondria. Multiple srimuli such as oxidants, Bax, Ca^{2+}, p53 protein and ceramide can trigger mitochondria to release caspase-activaqting proteins among which are cytochrome C and possibly other protein such as AIF. Cytochrome C is released from the intermembrane space of mitochondria into cytosol and activates caspases by binding to Apaf-1, including it associate with procaspase-9, thereby triggering caspase-9 activation and initiating the proteolytic cascade consisting of caspase-3 that culminates in apoptosis. Thesein, caspase-3 cleavages a variety of cellular substrates including ICAD and allows CAD (DFF40) to induce DNA laddering. Cells in which mitochondria have ruptured are at risk for death through a slower nonapoptotic mechanism resembling necrosis because of loss of the electrochemical gradient acoss the inner membrane ($\Delta\psi m$), production of ROS, and decling NAD levels and ATP production.

Mitochondria plays an important role in apoptosis and necrosis under GSH depletion

If mitochondria are pivotal in controlling cell life and death, then how do these organelles kill? At least three general mechanisms are known, and their effects may be interrelated, including (1) disruption of electron transport, oxidative phosphorylation, and ATP production; (2) release of protein that trigger activation of caspase family proteases; and (3) alteration of cellular reduction-oxidation (redox) potential. One consequence of the loss of electron transport should be a drop in ATP production (Fig 3). Although such a drop has been observed during apoptosis, it often occurs relatively late in the process. Indeed, ATP appears to be required for downstream events in apoptosis [39]. Mitochondrial impairment is occurred as an early event in the process of apoptosis induced by GSH depletion in neuronal cells [40]. Mitochondria are the major source of ROS including super oxide anion (O_2^-) production in cells. During transfer of electrons to molecular oxygen, an estimated 1 to 5% of electrons in the respiratory chain lose their way, most participating in formation of O_2^-. Super oxides and lipid peroxidation are increased during apoptosis induced by myriad stimuli. However, generation of ROS may be a relatively late event, occurring after cells have embarked on a process of caspase activation. A decrease in the capacity of mitochondria to reduce NAD(P), together with a decline in the NAD(P)H/NAD(P) redox couple, permeabilizes the inner mitochondrial membrane ($\Delta\psi m$). This favors the release of Ca^{2+} from the organelle and uncouples oxidative phosphorylation

and these effects lead to depletion of ATP. The reduced ATP levels also lead to further impairment of other Ca^{2+} regulation system, in the plasma membrane and the endoplasmic reticulum. In addition, the decrease in NAD(P)H compromises the activity of GSSG-reductase, and other protective enzymes, which further increases the deleterious effects of ROS [41]. An additional mechanism for the loss of NAD^+ and the deletion of ATP, involving DNA damage, has been proposed. In the case of single strand breaks in DNA, a poly(ADP-ribose) polymerase (PARP) is activated and involved in DNA excision repair. Activation of this PARP leads to a decrease in NAD^+ levels followed by depletion of ATP. It has been suggested that this is the basis of suicide mechanism of cell death to prevent the perpetuation of damaged DNA. Since overactivation of PARP may delete intracellular ATP and by itself promote the switch from apoptosis to necrosis, PARP cleavage by caspases during apoptosis and ensures appropriate execution of caspase-mediated apoptosis [42].

It has been reported that chromatin condensation in apoptosis depends on intracellular ATP levels or ATP synthesis [43]. Furthermore, the intracellular ATP level could be responsible for converting necrosis to apoptosis in oxidant-induced endothelial cell death [44]. Cell damage accompanies mitochondria membrane dysfunction not only in apoptosis but also in necrosis. A decrease in the mitochondrial membrane potential in the absence of a functional electron transport system results mainly from the leakage of protons through the membrane and proceeds apoptotic cell death [45].

As shown in Fig. 3, cytosolic cytochrome c forms an essential part of the vertebrate "apoptosome", which is composed of cytochrome c, apoptosis activating factor (Apaf)-1, and procaspase 9. The result is activation of caspase 9, which then processes and activates other caspases such as caspase-3 and caspase-7 to orchestrate the biochemical execution steps of apoptosis by cleaving many down stream substrate [46]. For example, the cleavage of ICAD (inhibitor of CAD) by caspase-3 results in the activation of CAD or DFF (DNA fragmentation factor), which leads to the formation of DNA ladder [34, 47]. These events are depended on intracellular ATP levels [48]. However, one should not quote caspase-independent cell death with necrosis. One mechanism may involve AIF. AIF, a mitochondrial flavoprotein with an oxidoreductase domain, was identified in a screen for mitochondria released proapoptotic factors [15]. AIF is localized to the mitochondrial intermembrane space in living cells and translocates to the cytoplasm and nucleus under certain apoptotic conditions. Various triggers of cell death cause release of AIF, which induces nuclear condensation and large-scale DNA fragmentation to fragments in a caspase-independent fashion [49].

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