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Review

Apoptosis and necrosis: Intracellular ATP level as a determinant for cell death modes

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Abstract

Apoptosis and necrosis are two distinct modes of cell death with respective morphological characteristics. However, apoptosis and some forms of necrosis must share common steps since both modes of cell death can be suppressed by the anti-apoptotic Bcl-2 protein and caspase inhibitors. Intracellular ATP levels have been implicated both *in vitro* and *in vivo* as a determinant of the cell's decision to die by apoptosis or necrosis.

Keywords: ATP, necrosis, apoptosis, mitochondria, bcl-2, caspase

Abbreviations: PI: propidium iodide ICE: interleukin-1B-converting enzyme

General considerations of cell death – apoptosis *versus necrosis*

Apoptosis, a mechanism of cell death, plays an important role in a variety of biological events, including morphogenesis, homeostatic maintenance of various tissues and removal of harmful cells (reviewed by Steller, 1995). Thus, dysregulated cell death can clearly lead to a variety of diseases (reviewed by Thompson, 1995). Apoptosis is characterized by chromatin condensation, nuclear fragmentation and formation of apoptotic bodies, which are phagocytosed by other cells (Wyllie *et al*, 1980). Apoptosis is tightly regulated by molecular mechanisms that appear to be evolutionary conserved (reviewed by Steller, 1995), and is therefore thought to be an active process in removing unnecessary cells. In most but not all forms of apoptosis, nuclear DNA is cleaved at internucleosomal sites. By contrast, cellular necrosis is defined by electron-lucent cytoplasm, mitochondrial swelling, loss of plasma membrane integrity without drastic morphological changes in nuclei. Necrosis has been considered a passive degenerative phenomenon induced by direct toxic or physical injuries, which most often occurs accidentally (Hawkins *et al*, 1972; Alison and Sarraf, 1994). Nuclear DNA is randomly cleaved as a consequence of cellular degeneration. Leakage of the cytoplasm through plasma membrane disruption induces cellular inflammatory responses. However, even at pathological regions or when cells are subjected to pathological reagents, apoptotic cells are often observed, indicating that accidental cell death can also proceed by apoptosis. Furthermore, dying cells with non-apoptotic morphological characteristics are observed during naturally occurring cell death. Thus, several forms of cell death which are currently defined by their morphology or by natural or accidental occurrence will soon be categorized according to their molecular mechanism.

Definitive assessment of apoptosis and necrosis

Because of the morphological definition of apoptosis, the best way to assess modes of cell death, apoptosis or necrosis is electron microscopy. However, it is not a very convenient way to determine the relative frequency of dead cells with apoptotic or necrotic features especially when dead cells in each mode simultaneously appear, for instance, when cells are subjected to hypoxia (Shimizu *et al*, 1995, 1996a). One of the convenient ways is to use fluorescence microscopy in conjunction with different cell staining (Shimizu *et al*, 1996a). Cells can be double-stained by calcein-AM (green) which stains whole cells except vacuole, associated with necrosis, irrespective of membrane integrity, and propidium iodide (PI)(pink) which stains only nuclei in cells which disrupted plasma membrane integrity, a hallmark of necrosis. The stained cells are visualized under a confocal microscope. Alternatively, cells can be stained by PI (red) and Hoechest 33342 (blue). Hoechst 33342 dye stains nuclei of all cells whereas PI stains only cells with disrupted membrane integrity and visualized under a nonconfocal fluorescence microscope. How apoptotic and necrotic cells look by respective staining procedure are summarized in Table 1 and the representative photographs shown in Figure 1.

The common machinery of apoptosis

Apoptosis can be induced by a variety of stimuli, including depletion of growth factors, hormones, heat shock, γ-irradiation and crosslinking of Fas antigen (reviewed by Thomspon, 1995). Apoptotic signal transduction pathways activated by various treatments converge into a common pathway, which is driven by ICE/Ced-3 family protease, designated caspases (reviewed by Martin and Green, 1995; Alnemri *et al*, 1996), and negatively regulated by anti-cell death proteins such as the Bcl-2/Ced-9 family (reviewed by Cory, 1995) and the IAP family (Clem and Miller, 1994; Hay *et al*, 1995). However, the biochemical basis of the functions of these anti-apoptotic proteins remains unknown.

A number of mammalian *ice*-related genes have been identified, and ICE (caspase-1)(-like), Ich-1 (caspase-2)

(-like), and CPP32/Yama (caspase-3)(-like) proteases have been implicated in apoptosis, primarily based on inhibition of apoptosis by their synthetic or natural inhibitors (reviewed by Kumar, 1995). Caspases are initially produced as inactive precursors (zymogens) which are subjected to specific proteolytic cleavage for their activation. Caspases have been suggested to constitute a protease cascade, based on observations that they cleave other members of the family *in vitro* to activate them (reviewed by Martin and Green, 1995). Recently, sequential activation of caspase 1(-like) and caspase 3(-like) proteases has been shown to be required for apoptosis *in vivo*, which is induced by different stimuli including Fas stimulation, VP16 and calcium ionophore (Enari *et al*, 1996; Shimizu *et al*, 1996c). Although several

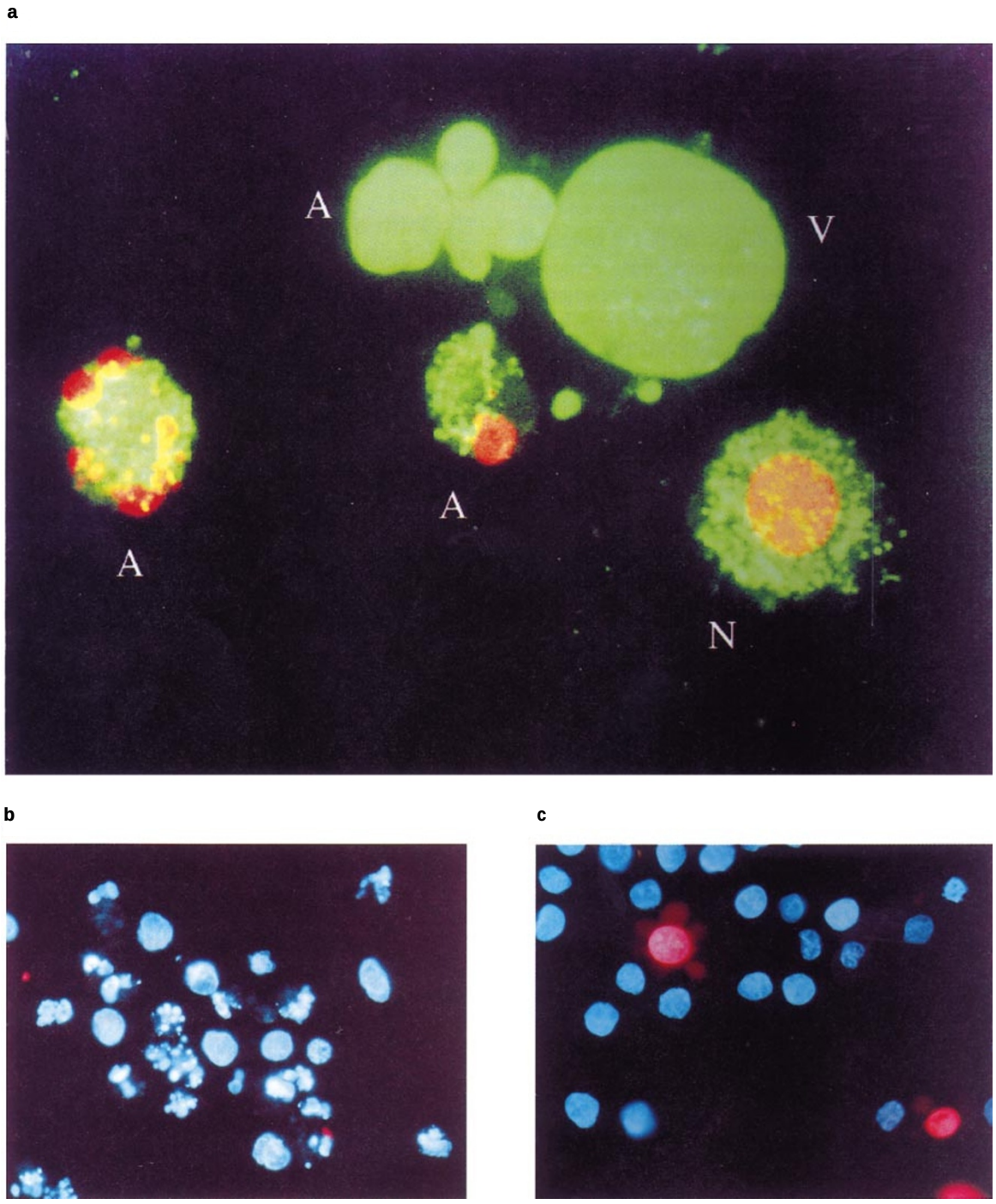

Figure 1 Assessment of apoptosis and necrosis using fluorescence microscopy (**a**) confocal fluorescence micrograph of PC12 cells treated with hypoxia after being stained with calcein-AM and PI (modified from Shimizu *et al*, 1996a). A, N and V indicate apoptotic, necrotic and viable cell, respectively. (**b,c**) nonoconfocal fluorescence micrographs of apoptotic (**b**) and necrotic Hela cells (**c**) stained with PI and Hoechst 33342

Table 1 Cell death modes assessed by fluorescence staining

	Confocal		Nonconfocal	
	Calcein-AM (green)		PI (red)	Hoechst 33342 (blue)
	Cell shape	Vacuole	Nuclei	Nuclei
Viable	R*	–	Not stained	R
Necrosis	R	+	R	R
Early apoptosis	F**	–	Not stained	F
Terminal apoptosis	F	–	F	F

*round, **fragmented.

cellular victims for caspases such as poly (ADP-ribose) polymerase, actin and lamins have been described, an essential role for the cleavage of victims in apoptosis has not been directly demonstrated. True targets for caspases remains to be elucidated.

Inhibition of some forms of necrosis by Bcl-2 and caspase inhibitors

Although Bcl-2 and caspase inhibitors had been thought to specifically inhibit apoptosis, preventive effects of Bcl-2 on necrosis were occasionally observed (Strasser *et al*, 1991; Kane *et al*, 1993; Shimizu *et al*, 1995). More convincing evidence for the role of Bcl-2 in necrosis was provided by the demonstration that Bcl-2 and its relative Bcl-x_L inhibit necrotic cell death induced by oxygen depletion (Shimizu *et al*, 1996a, e), respiratory chain inhibitors such as KCN and antimycin A (Shimizu *et al*, 1996b) or by glutathione depletion (Kane *et al*, 1995), as confirmed morphologically by electron microscopy. Such necrotic cell death was also retarted by caspase inhibitors including tetrapeptide inhibitors and a serpin, CrmA derived from cowpox virus (Shimizu *et al*, 1996b–e). These results indicate that apoptosis and some forms of necrosis share common steps, at least common target sites for Bcl-2/Bcl-x_L and for caspases (Figure 2).

Anti-apoptotic Bcl-2 localizes in multiple membrane compartments, including the nuclear envelope, endoplasmic reticulum and mitochondrial membranes (Hockenbery *et al*, 1990; Monaghan *et al*, 1992; Jacobson *et al* 1993; Akao *et al*, 1994). However, the subcellular compartment in which Bcl-2 acts to prevent cell death has not been precisely identified. Accumulating evidence indicates that overexpressed Bcl-2 prevents the activation of caspases in apoptosis and hypoxia-induced necrosis, suggesting that Bcl-2 prevents cell death by acting upstream of the activation of caspases (Chinnaiyuan *et al*, 1996; Boulakia *et al*, 1996; Shimizu *et al*, 1996c; Armstrong *et al*, 1996). Several models for Bcl-2's ability to block the activation of the caspase cascade have been proposed: (1) Bcl-2 prevents mitochondrial dysfunction such as membrane potential ($\Delta\Psi$) loss and membrane permeability transition (PT) (Zamzami *et al*, 1995a; Shimizu *et al*, 1996d) which allows the release of mitochondrial apoptogenic factors such as cytochrome c (Liu *et al*, 1996) and AIF (Susin *et al*, 1996) (discussed by G Kroemer, 1997) (2) Bcl-2 sequesters caspases by interaction through a ced-4-like protein

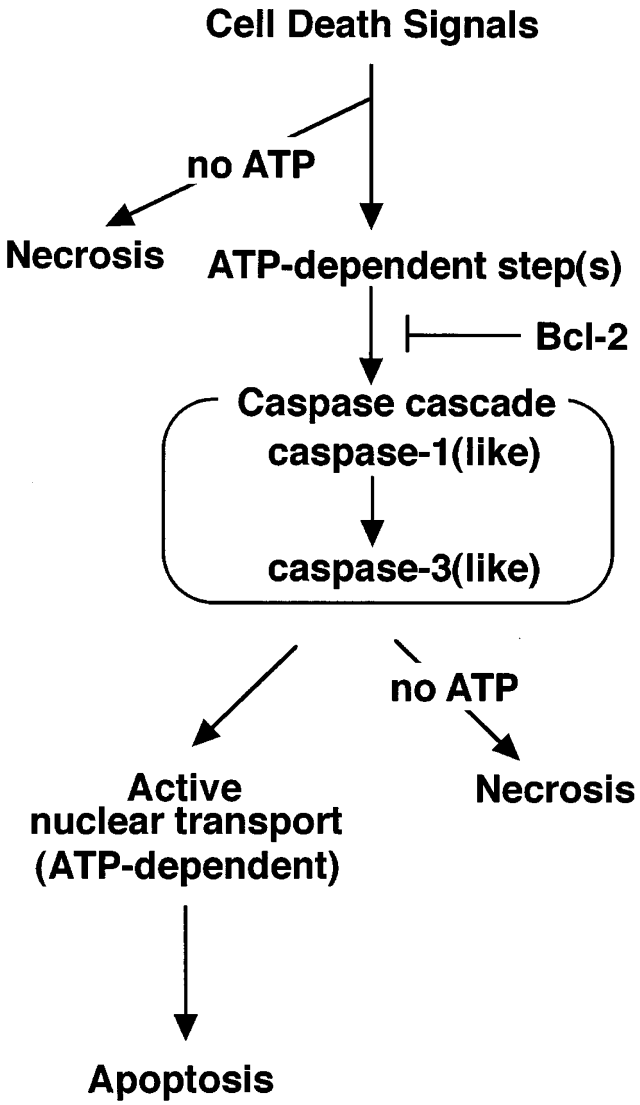

Figure 2 Apoptosis and some forms of necrosis share a common signal transduction pathway

(Chinnaiyan *et al*, 1997) and (3) Bcl-2 inhibits the $\Delta\Psi$-independent release of cytochrome c which leads to the activation of the caspases (Yang *et al*, 1997; Kluck *et al*, 1997).

ATP, an intracellular determinant of cell death by apoptosis *versus necrosis*

What might determine whether cells die by apoptosis or necrosis? One evident physiological difference in cells undergoing apoptosis versus necrosis is in the levels of intracellular ATP. Since ATP depletion itself induces necrosis, indicating that necrosis does not require intracellular ATP, studies were done to directly address the question whether apoptosis is ATP-dependent (Eguchi *et al*, 1997). Depletion of intracellular ATP by incubating cells in glucose-free medium to halt glycolysis, in the presence of the mitochondrial F_0F_1-ATPase inhibitor

oligomycin, completely blocked apoptosis induced by Fas stimulation, VP16, dexamethasone or calcium ionophore, and ATP supplied through either glycolysis or mitochondrial oxidative phosphorylation restored the apoptotic cell death pathway, indicating that apoptosis is ATP-dependent (Eguchi *et al*, 1997). The same conclusion was also reached by Leist *et al*, (1997) (discussed by Nicotera and Leist, 1997).

Shimizu *et al*, (1995d), measured intracellular ATP levels during cell death, and shown that intracellular ATP levels remain unchanged until the very end of apoptotic process such as disruption of plasma membrane. Since it has been reported that loss of mitochondrial membrane potential which halts mitochondrial ATP production, is an early step in apoptosis (Zamzami *et al*, 1995b; Shimizu *et al*, 1996d), intracellular ATP required for the rest of apoptotic processes must be provided from glycolysis.

ATP-dependent steps both upstream and downstream of the activation of executioner caspases

ATP-dependent steps exist both upstream and downstream of caspase 3(-like) protease activation in the apoptotic signal transduction pathway activated by various apoptotic stimuli (Figure 2). Which step in apoptosis is ATP-dependent? Because apoptosis is characterized by morphological changes in the nucleus, the apoptotic death signals must be transmitted from the cytoplasm to the nucleus. It has recently been shown that active nuclear transport, which is responsible for transfer of relatively large molecules across the nuclear membrane through nuclear pores, is essential for Fas-mediated and recombinant active caspase 3-induced apoptosis (Yasuhara *et al*, 1997), indicating that a cytoplasmic apoptotic effector(s) with a relatively large molecular weight must be transferred to the nucleus in other to induce apoptotic changes in the nucleus. Some caspases or their target(s) are likely translocated from the cytoplasm to the nucleus. Translocation of some caspases into the nucleus would be consistent with the observations that nuclear proteins such as lamins and poly(ADP)-ribose polymerase are cleaved by caspases (Martin and Green, 1995). It has recently been shown using a reconstituted system with naked nuclei and death lysates that the presence of ATP is necessary for chromatin condensation and nuclear fragmentation but not for DNA fragmentation (Kass *et al*, 1996). Note that apparent ATP-independence of DNA fragmentation does not necessarily indicate that cytosolic effectors for DNA fragmentation might gain access to the nuclear matrix in the absence of ATP because nuclear transport with naked nuclei does not require the ATP-dependent mechanism any more.

Given that the Fas-initiated death signal is transferred via caspase-8 recruited into the Fas receptor complex to the caspase cascade that includes caspase-3 (reviewed by Nagata, 1997), the existence of an ATP-dependent step(s) upstream of the activation of caspase-3 might mean that the signal transduction pathway from Fas to the caspase cascade is regulated by protein phosphorylation.

Conversion of apoptotic signals to necrotic signals under ATP-depleting condition

Treatment with calcium ionophore induces apoptosis under ATP-supplying conditions but necrotic cell death under ATP-depleting conditions (Eguchi *et al*, 1997), indicating that ATP levels are a determinant of how cell death is manifested, and apoptotic signals might be converted to necrotic signals in at least some cases. Note that although chemical hypoxia- and hypoxia-induced necrotic cell death require the activation of caspases, ATP depletion after the Fas-mediated activation of caspase-3(-like) proteases failed to convert efficiently Fas-initiated signals to necrotic signals, raising the possibilty that different caspases might be activated by the respective cell death stimuli. Alternatively, necrotic cell death fate might also depend on additional signals that are co-operated only by some cell death stimuli. Since activation of caspase-3 is not observed in calcium ionophore-induced necrosis under ATP-deleting condition, some forms of necrosis might proceed independent of caspase activation simply through cellular collapse (Figure 2) although activation of another caspases might be involved. It should be noted that in the case of hypoxia- and chemical hypoxia-induced necrosis, the death signal flows through caspase activation because the cell death is suppressed by caspase inhibitors (Shimizu *et al*, 1996b – e). Thus, necrosis might be classified into two categories, one that shares common steps with apoptosis and one that does not.

Implications

ATP-dependence of apoptosis but not necrosis might explain the frequent appearance of necrotic cells mixed with apoptotic cells in pathological areas *in vivo*, such as the center of solid tumors (reviewed by Ledda-Columbano and Columbano, 1991 and by Szende *et al*, 1991) and ischemic nervous tissues (reviewed by Chaniaut-Marlangue *et al*, 1996). In areas where blood flow is limited, intracellular ATP supplies are rapidly exhausted due to insufficient oxygen and rapid consumption of glucose, inhibiting apoptosis and inducing necrotic cell death. Thus, *in vivo*, intracellular ATP levels appear to be one of the factors that determine cell death fate by apoptosis or necrosis.

When glucose is provided in a medium, respiratory chain inhibitors, depending on cell lines or cell types, induce apoptosis or necrosis (Shimizu *et al*, 1996a). The distinction is due to the cell's dependence on respiration to maintain intracellular ATP levels. Cells depending more on mitochondria for ATP, die by necrosis because of energy crisis and cells with larger contribution of glycolysis die by apoptosis because of lowered intracellular ATP levels which are still sufficient for apoptosis.

As often noted, another factor affecting modes of cell death might be the concentrations of toxic reagents used; the same reagent induces apoptosis at lower concentrations but kills cells by necrosis at higher concentrations probably due to the rapid collapse of cellular integrity including plasma membrane disruption, a large calcium influx (extensively discussed by Nicotera, this issue) as well as intracellular ATP depletion.

Cells that die pathologically but by apoptosis do not generate any warning to the host through inflammation and might be cleared by normal apoptotic mechanisms of engulfment. Small-scale pathological cell death might not merit a warning to the host or might be a sacrifice for having apoptotic mechanisms.

Since apoptosis is tightly regulated at genetic levels, optimism exists that diseases caused by enhanced apoptosis might be treated by inhibiting molecular processes with drugs or through some gene therapy. However, since at least some forms of necrosis are rescued by anti-apoptotic agents, the question of whether the cells of interest die by apoptosis or necrosis might be less important than knowledge about the mechanisms underlying the cell death.

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