The role of calcium in the regulation of apoptosis

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Abstract: The recognition that apoptosis is regulated by an evolutionarily conserved set of polypeptides from the nematode *Caenorhabditis elegans* to humans suggests that a conserved set of biochemical mechanism(s) may also be involved in the response. Work from a number of independent laboratories suggests that alterations in cytosolic Ca^{2+} homeostasis represent one such candidate mechanism, and molecular targets for Ca^{2+} are now being identified. This review will summarize what is known about the role of Ca^{2+} in the regulation of apoptosis and discuss how Ca^{2+} might interact with some of the other biochemical signals implicated in cell death. *J. Leukoc. Biol.* 59: 775–783; 1996.

Key Words: *endonuclease · oxidative stress · BCL-2*

INTRODUCTION

Apoptosis (programmed cell death) is a highly regulated process of selective cell deletion involved in development, normal cell turnover, hormone-induced tissue atrophy, cell-mediated immunity, tumor regression, and a growing number of pathological disorders (typified by AIDS and Alzheimer's diseases) [1, 2]. The response is characterized by a series of morphological alterations, including plasma and nuclear membrane blebbing, organelle relocalization and compaction, chromatin condensation, and the formation of membrane-enclosed structures termed apoptotic bodies that are extruded into the extracellular millieu [2, 3]. Uptake of this cellular debris is also carefully controlled. Apoptotic cells and bodies are specifically recognized and cleared by neighboring epithelial cells and professional phagocytic cells (macrophages) before their contents can be released into the extracellular millieu, thereby allowing for cell death to occur in the absence of inflammation [3].

Biochemically, apoptosis has historically been characterized by endogenous endonuclease activation, resulting first in the production of domain-sized large (50–300 kilobase) DNA fragments [4–6] and subsequently into oligonucleosomal cleavage products commonly referred to as DNA ladders [7]. It appears that the latter are derived from the former, and there is also some evidence that the initial generation of kilobase-sized fragments is mediated by a different enzymatic activity from the one that forms the DNA ladders (due to differences in divalent cation requirements for activity) [8]. In addition to endonuclease activation, more recent work has demonstrated that a family of cysteine proteases homologous to the *Caenorhabditis elegans* cell death gene *ced-3* and human interleukin-1-converting enzyme (ICE) are also critically involved in the response, as inhibitors of these enzymes block both endonuclease activation and cell death [9–16]. The important substrates for these proteases remain largely unidentified but include poly(ADP-ribose) polymerase (PARP), the lamins, and a viral inhibitor of their activity (baculovirus p35) [10, 17–20].

At the molecular level, apoptosis is regulated by a growing list of oncogenes (*bcl-2*, *myc*, *ras*, *abl*, *fos*) and tumor suppressor genes (*p53*, *Rb*) [1]. However, the family of polypeptides homologous to the *bcl-2* appear to play particularly important roles. Regulation of apoptosis susceptibility is their only established function to date. One class of *bcl-2* homologues (including *bcl-2*, *bcl-x_L*, *mcl-1*, and several viral proteins) suppress apoptotic cell death, while another group (*bax*, *bcl-x_s*, and *bak*) promotes apoptosis sensitivity. Korsmeyer's laboratory has provided a possible mechanistic explanation for these differential effects on apoptosis with the observation that the BCL-2 family members form dimers that can apparently impact the process in opposite fashions; when BCL-2:BAX heterodimers predominate, cells are resistant to apoptosis, whereas a preponderance of BAX:BAX homodimers promotes susceptibility to cell death [21]. Most importantly, overexpression of BCL-2 or BCL-X_L blocks apoptosis induced by very diverse stimuli, including growth factor withdrawal, tumor necrosis factor, engagement of the Fas antigen, ionizing radiation, oncogenes such as myc, and chemical chemotherapeutic agents [1], strongly suggesting that they function at a downstream site within the apoptotic pathway that is proximal to the effector machinery [proteases and nuclease(s)]. Moreover, the apoptosis-regulatory functions of BCL-2 and its homologues are evolutionarily conserved, as the *C. elegans* cell death suppressor *ced-9* is a structural and functional homologue of human *bcl-2* [22, 23].

Abbreviations: ICE, interleukin-1-converting enzyme; PARP, poly(ADP-ribose) polymerase; IL-3, interleukin-3; NK cells, natural killer cells; IP_3, inositol trisphosphate; NS, nuclear scaffold; $\Delta\Psi$, mitochondrial membrane potential.

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Together, these observations have suggested to many investigators that BCL-2 regulates a central biochemical signal in the pathway to cell death, the identity of which is still unknown. However, several strong candidates are emerging. Work from our laboratory and others over the past 5 years has demonstrated that alterations in intracellular Ca^{2+} homeostasis are commonly involved in promoting apoptosis and more recent work suggests that one aspect of BCL-2 function involves preventing these alterations. Here we will discuss the role of Ca^{2+} in regulating apoptosis and compare it with other general mediators of apoptotic cell death.

EVIDENCE FOR THE INVOLVEMENT OF Ca^{2+} ALTERATIONS IN APOPTOSIS

Early studies by Kaiser and Edelman provided the first evidence that increases in intracellular Ca^{2+} might be involved in triggering apoptosis. Working with immature thymocytes, the authors showed that glucocorticoid-stimulated apoptosis is associated with enhanced Ca^{2+} influx [24]. We have since confirmed that glucocorticoids induce cytosolic Ca^{2+} elevations in thymocytes via Ca^{2+} influx [25]. However, intracellular Ca^{2+} storage sites may also be affected. For example, the Ca^{2+} pool located in the endoplasmic reticulum is depleted in a lymphoid cell line in response to glucocorticoid treatment [26], and a similar phenomenon has been documented in an interleukin-3 (IL-3)-dependent myeloid cell line following IL-3 withdrawal [27]. Circumstantial evidence suggests that the mitochondrial Ca^{2+} pool may also be affected [28], as mitochondrial membrane potential drops very early during apoptosis [29–31], and it is well known that the maintenance of mitochondrial Ca^{2+} homeostasis is dependent upon mitochondrial membrane potential [28]. Apoptosis in other systems also appears to involve alterations in Ca^{2+} compartmentalization. For example, rapid, sustained Ca^{2+} increases precede the cytolysis of the targets of cytotoxic T lymphocytes [32] and natural killer (NK) cells [33]. In developing T lymphocytes high-affinity engagement of the T cell receptor induces apoptosis [34–37] that involves a sustained Ca^{2+} elevation [37, 38].

There appear to be at least two general mechanisms involved in promoting Ca^{2+} increases in apoptotic cells (**Fig. 1**). The first of these is directly analogous to the mechanism involved in the promotion of Ca^{2+} increases in cells exposed to growth factors or other agonists. For example, T cell receptor engagement leads to a sustained increase in the cytosolic Ca^{2+} concentration in mature T cells that is required for stimulation of autocrine growth factor (IL-2) production and proliferation. The mechanism underlying the increase involves protein tyrosine kinase activation, activation of the γ-isoform of phospholipase C, phosphoinositide hydrolysis leading to the production of inositol trisphosphate (IP_3), and mobilization of Ca^{2+} from the endoplasmic reticulum and extracellular millieu. A similar mechanism leads to sustained Ca^{2+} increases in immature thymocytes following TCR engagement, but in this case these signals promote cell death [37, 39]. Similarly, surface antigen receptor engagement on B cells leads to Ca^{2+} increases that promote cell death [40–43]. Thus, in these examples of apoptosis, Ca^{2+} increases occur via a controlled, physiological mechanism that is also utilized in alternative responses such as cellular activation leading to proliferation. Interestingly, in mature T and B cells and various cell lines, Ca^{2+} may act to promote apoptosis via activation of an autocrine suicide pathway involving Fas and Fas ligand expression.

Work from our laboratories and others has revealed another mechanism that is involved in promoting sustained cytosolic Ca^{2+} increases in apoptotic cells. It is well known that cytosolic Ca^{2+} concentration is maintained at roughly 100 nM in resting cells, whereas the concentrations in the extracellular millieu, and the endoplasmic reticulum (ER), are much higher (in the millimolar range). Early work on the biochemical mechanisms underlying the cytotoxicity of agents that generate reactive oxygen species in cells (oxidative stress) indicated that the Ca^{2+} transport systems localized to the ER, mitochondria, and plasma membrane can be damaged by oxygen radicals [44]. This leads to diffusion of Ca^{2+} down its concentration gradient, a disruption of intracellular Ca^{2+} homeostasis, and sustained Ca^{2+} increases. Oxidative stress is now known to be commonly

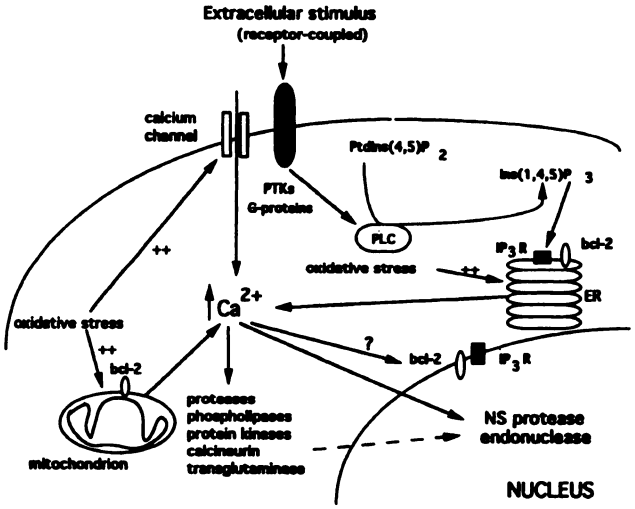

Fig. 1. Targets for Ca^{2+} in the regulation of apoptosis. Extracellular agonists, calcium ionophores, or the endoplasmic reticular Ca^{2+} ATPase antagonist thapsigargin are capable of triggering sustained Ca^{2+} increases that mediate apoptosis in diverse model systems. Alternatively, recent work suggests that oxidative stress can disrupt intracellular Ca^{2+} homeostasis, possibly via oxidation of critical sulfhydryls present in the Ca^{2+} translocases located in the plasma membrane, mitochondria, and endoplasmic reticulum. Of interest is the fact that members of the BCL-2 family of apoptosis suppressors are localized to several intracellular Ca^{2+} regulatory sites, including the mitochondria, endoplasmic reticulum, and nucleus. Calcium elevations most likely exert their effects via activation of both cytosolic and nuclear targets, the latter of which include a Ca^{2+}-dependent protease associated with the nuclear scaffold (NS protease) and the endonuclease. Abbreviations: $PtdIns(4,5)P_2$, phosphatidylinositol-(4,5)-bisphosphate; $Ins(1,4,5)P_3$, phosphatidylinositol (1,4,5)-trisphosphate; IP_3R, receptor for $Ins(1,4,5)P_3$.

involved in apoptosis [45–49], and it is therefore possible that oxidative disruption of intracellular Ca^{2+} homeostasis is involved in these systems. Supporting this idea, we have recently shown that the glucocorticoid-induced Ca^{2+} increase observed in thymocytes is blocked by antioxidants [50], and Kroemer's laboratory has presented evidence that oxidative stress leads to disruption of mitochondrial Ca^{2+} stores [29].

Direct evidence that Ca^{2+} increases can mediate apoptotic endonuclease activation and cell death has been obtained from experiments with intracellular Ca^{2+} buffering agents and extracellular Ca^{2+} chelators. We [25, 33, 37, 39, 51–53] and others [54, 55] have shown that these agents can inhibit both DNA fragmentation and death in apoptotic cells. The Ca^{2+}-dependent regulatory cofactor calmodulin may link these Ca^{2+} alterations to the effector machinery, as we and others have shown that calmodulin antagonists can interfere with apoptosis in some of these systems [25, 56] and increases in calmodulin expression are linked to apoptosis in glucocorticoid-treated thymoma cells [56] and in prostatic epithelial cells following withdrawal of androgen [57]. Independent evidence for the involvement of Ca^{2+} influx in the triggering of apoptosis has come from studies with specific Ca^{2+} channel blockers, which abrogate apoptosis in the regressing prostate following testosterone withdrawal [58] and in pancreatic β cells treated with serum from patients with type I diabetes [59].

Other support for the involvement of Ca^{2+} in apoptosis comes from the observation that agents which directly mobilize Ca^{2+} can trigger apoptosis in diverse cell types. Early work by Kaiser and Edelman demonstrated that the cytolytic effects of glucocorticoids on lymphoid cells can be mimicked by treating the cells with Ca^{2+} ionophores [60]. Subsequently, Wyllie and co-workers demonstrated that Ca^{2+} ionophores cause endonuclease activation as well as many of the morphological changes that are typical of apoptosis in thymocytes [61]. Calcium ionophores also trigger apoptosis in prostate tumor cells [58] and in nonmetastatic melanoma lines [D. J. McConkey, unpublished results]. Independent evidence for the general relevance of this mechanism has come from studies with the endoplasmic reticular Ca^{2+} ATPase inhibitor thapsigargin, the product of the plant, *Thapsa garganica*, which can also trigger all of the morphological and biochemical events of apoptosis in thymocytes [62] and some other cell types [63–65].

A final argument for a central role for Ca^{2+} in regulating apoptosis comes from recent and ongoing work on the biochemical mechanisms of apoptosis suppression by the BCL-2 oncoprotein. The possible relationship between Ca^{2+} and BCL-2 was first suggested by the work of Baffy and colleagues, who showed that BCL-2 can block the depletion of the endoplasmic reticular Ca^{2+} pool in transfectants of an IL-3-dependent cell line (32D) [27]. Interestingly, these authors also demonstrated that constitutive levels of Ca^{2+} in mitochondria (measured following treatment with an uncoupler that promotes rapid and selective depletion of this intracellular Ca^{2+} store) were significantly lower in BCL-2-expressing cells compared with vector control transfectants, consistent with the notion that BCL-2 may also regulate Ca^{2+} compartmentalization in mitochondria. More recently, Lam and co-workers have shown that overexpression of BCL-2 interferes with thapsigargin-induced Ca^{2+} mobilization from the ER in the WEHI7.2 T lymphoma cell line, an effect that is associated with preservation cell viability [66]. Precisely how BCL-2 regulates intracellular Ca^{2+} is still unclear, although given its co-localization with Ca^{2+} transport sites in mitochondria, the ER, and the nuclear envelope [67], a direct effect of BCL-2 on Ca^{2+} channel(s) is possible. Alternatively, given the tight interrelationship between Ca^{2+} and oxidative stress, BCL-2 could be influencing Ca^{2+} homeostasis via effects on cellular redox status [46, 68].

EFFECTS OF ALTERED Ca^{2+} HOMEOSTASIS: COUPLING TO THE EFFECTOR PATHWAY

An important aspect of ongoing research involves defining the biochemical consequences of Ca^{2+} mobilization in apoptotic cells. At present there are two basic theories to explain how these alterations might trigger apoptosis. In one, depletion of intracellular stores and possibly influx of Ca^{2+} across the plasma membrane promote a sustained Ca^{2+} increase that acts as a signal for apoptosis, perhaps in part by activating key catabolic enzymes that make up the effector machinery. In the second, it is not the Ca^{2+} increase but the emptying of intracellular Ca^{2+} stores that triggers apoptosis, perhaps by disrupting intracellular architecture and allowing key elements of the effector machinery to gain access to their substrates. These models are certainly not mutually exclusive. Evidence for both models will be presented below, but it should be emphasized at the outset that definitive proof for either one is lacking at present.

POSSIBLE TARGETS FOR Ca^{2+} ELEVATIONS

Signal transduction intermediates

As noted above, apoptosis in a number of different model systems is associated with sustained increases in the cytosolic Ca^{2+} concentration, and in some of these systems DNA fragmentation and cell death can be blocked by intracellular Ca^{2+} buffering agents. Given the clear involvement of analogous Ca^{2+} increases in signal transduction pathways regulated by extracellular ligands, it is certainly possible that they regulate apoptosis via similar mechanisms involving activation of Ca^{2+}-dependent protein kinases and/or phosphatases leading to alterations in gene transcription. Experiments with the immunosuppressant cyclosporin A support this model for how Ca^{2+} elevations may be coupled to downstream events in apoptosis. Cyclosporin A binds a family of cytosolic receptors termed cyclophilins, and in so doing forms a composite molecular surface that binds to and inhibits the Ca^{2+} calmodulin-de-

pendent protein serine/threonine phosphatase, calcineurin [69]. Studies by several independent laboratories have shown that cyclosporin A can block Ca^{2+}-dependent apoptosis in lymphoid model systems [35, 70–72], indicating that calcineurin activation may be required for these responses. Our recent work confirms that cyclosporin A and FK506 block Ca^{2+}-stimulated apoptosis in T cell hybridomas but they are without effect on Ca^{2+}-dependent apoptosis in immature rodent thymocytes in vitro [S. Jiang, S. C. Chow, S. Orrenius, unpublished results]. Induction of the orphan steroid receptor Nur77 and the Fas ligand represent at least two of the confirmed molecular targets of calcineurin in mature T cells and T cell hybridomas [73, 74]. The involvement of calcineurin in Ca^{2+}-stimulated apoptosis could also potentially explain in part the sensitivity of various apoptotic pathways to inhibition by calmodulin antagonists.

Ca^{2+}-activated proteases

There is some evidence that Ca^{2+}-sensitive protease(s) might represent direct targets for Ca^{2+} elevations in apoptosis. Recent work has shown that the Ca^{2+}-dependent neutral protease, calpain, is rapidly activated in T lymphocytes following treatment with glucocorticoids or exposure to γ-irradiation, and that calpain antagonists can block DNA fragmentation associated with the response [75]. Similarly, Henkart's group has demonstrated that some (but not all) pathways of apoptosis in mature T lymphocytes can be inhibited by calpain antagonists [76, 77]. Thus, investigators are now searching for calpain substrates that may play important roles in apoptotic cell death. The cytoskeletal protein, fodrin, is at least one substrate for calpain that is cleaved in T cells following treatment with glucocorticoids or engagement of the Fas antigen [78]. Similarly, we have recently obtained evidence that the cytoskeletal protein, vimentin, is also cleaved in apoptotic cells by a calpain-sensitive mechanism [J. Kiefer et al., unpublished observation]. Precisely how fodrin and vimentin cleavage participate in the apoptosis effector mechanism is unclear, although they may be involved in cellular shrinkage, membrane blebbing, or other structural alterations associated with apoptosis.

Other Ca^{2+}-sensitive proteases may also participate in the process. Previous work has demonstrated that incubation of isolated nuclei in the presence of Ca^{2+} promotes the rapid degradation of a family of nuclear matrix proteins, the lamins [79, 80]. The protease responsible for lamin cleavage in this system is directly associated with the nuclear matrix and is activated by Ca^{2+}. In parallel, independent work from several laboratories has shown that lamins are also degraded in cells undergoing apoptosis [19, 81–83], and in thymocytes lamin cleavage occurs via a Ca^{2+}-dependent mechanism [82], inspiring investigators to investigate the possible involvement of the lamin protease in the response. Thus, Zhivotovsky and colleagues in one of our laboratories have found that a specific peptide inhibitor of the putative lamin protease [also known as nuclear scaffold (NS) protease] blocks cellular shrinkage and DNA fragmentation in thymocytes exposed to thapsigargin and glucocorticoids, but not in cells treated with etoposide, a cancer chemotherapeutic agent that acts via induction of DNA damage. We have also found that inhibitors of this protease selectively block both lamin cleavage and DNA fragmentation in isolated nuclei [D. J. McConkey, unpublished results]. Importantly, however, the NS protease is not the only protease that can cleave the lamins, as indicated by recent work by Lazebnik et al. [84]. In their model, isolated nuclei from untreated cells are incubated with extracts obtained from cells arrested at the S phase (and committed to apoptosis), released, and lysed at the M phase of the cell cycle. These S/M extracts promote all of the nuclear alterations observed in whole cells undergoing apoptosis [19]. Incubation of S/M extract-treated nuclei with an inhibitor of the ICE/ced-3 family of cysteine proteases blocks lamin cleavage, whereas treatment with an inhibitor of the lamin protease does not [84]. Moreover, in this system lamin cleavage, chromatin condensation, and DNA fragmentation do not require exogenous Ca^{2+}. Whether Ca^{2+} is involved in generating the apoptosis-promoting activity found within these S/M extracts is not clear at present. In addition, whether BCL-2 is capable of blocking the nuclear alterations induced by the S/M extracts is also not known, and it is therefore possible that the activity present in them is an irreversibly activated component of the effector machinery (i.e., an ICE protease) that is no longer subject to Ca^{2+} regulation.

Ca^{2+}-activated endonuclease(s)

As introduced above, endonuclease activation resulting in the formation of oligonucleosome-length DNA fragments (DNA ladders) remains the most characteristic biochemical feature of apoptotic cell death. Early work by Hewish and Burgoyne [85] and later by Vanderbilt and colleagues [86] demonstrated the presence of a Ca^{2+}/Mg^{2+}-dependent enzyme activity capable of generating characteristic apoptotic chromatin cleavage patterns within nuclei of various cell types. Subsequent work by Cohen and Duke [87], and Wyllie and co-workers [61], implicated this activity in the DNA fragmentation observed in thymocytes undergoing apoptosis, and it is now thought that it mediates DNA fragmentation in a variety of other model systems as well. Search for and purification of potential apoptotic nucleases were undertaken by several laboratories. Thus, Gaido and Cidlowski [88] described a low-molecular-weight nuclease (NUC18) with Ca^{2+} and Mg^{2+} dependence activity in apoptotic lymphoid cells in response to several kinds of apoptotic stimuli. (Interestingly, the purified NUC18 shares amino acid sequence homology with cyclophilin and human recombinant cyclophilin A has biochemical and pharmacological properties identical to native NUC18 [89].) This enzyme has a nuclear localization in a variety of cell types, and the nuclease activity of these cells could be induced by several signal transduction pathways that are known to mediate apoptosis. NUC18 is also

present in untreated thymocytes in precursor form or as part of a higher-molecular-mass complex (>100 kDa), suggesting that the enzyme is maintained in an inactive complex from which the nuclease dissociates in response to apoptotic signals. Although the precise mechanism of liberation of active enzyme from its precusors is unknown, an attractive possibility is that it may involve proteolysis.

The Ca^{2+}-dependent endonuclease DNase I is another excellent candidate apoptotic nuclease [90]. Addition of the enzyme to isolated nuclei and other reconstitution systems promotes the formation of DNA strand breaks that possess the same 5′-PO_4 and 3′-OH end groups found in DNA fragments isolated from apoptotic cells. Although the enzyme is localized within the rough ER, the Golgi complex, and small (secretory) vesicles in viable cells, it is also found within the perinuclear space of apoptotic cells, and it is possible that structural alterations in the ER and/or nuclear envelope associated with apoptosis may promote the entry of DNase I into the nucleus (see below). A similar mechanism may promote entry of an ER-localized fraction of the NS lamin protease into the nucleus. Several other proteins with Ca^{2+}/Mg^{2+} endonuclease activity have been isolated [91–94], but to date proof that any one of these activities is directly involved in oligonucleosomal DNA fragmentation in apoptosis is lacking.

Transglutaminase activation

Transglutaminases are a group of Ca^{2+}-dependent enzymes that catalyze the posttranslational coupling of amines (including polyamines) into proteins and the cross-linking of proteins via gamma glutamyl lysine bridges when the amine is a peptide-bound lysine residue. Tissue transglutaminase has been implicated in a number of physiological processes, including cross-linking of integral plasma membrane proteins with the cytoskeleton. Recent work indicates that tissue transglutaminase is also involved in induction of apoptosis [95, 96]. Expression of transglutaminase mRNA and protein levels increase markedly in dying cells. The enzyme appears to be activated by elevations of the cytosolic Ca^{2+} concentration, which are involved in apoptosis in many different systems. Isolation of apoptotic bodies from a number of different tissues has shown that they are resistant to dissolution by detergents and chaotrophic agents; this may in part be explained by the fact that surface polypeptides in these structures are cross-linked via gamma glutamyl lysine isopeptide bonds [97]. The resistance of these structures to proteolysis appears to allow them to accumulate, and they can be detected in the media of cell cultures containing high rates of apoptotic cell death [98]. Isodipeptide can also be detected in normal plasma, and its concentration increases following induction of apoptosis in various organs, including the thymus and liver.

The role of transglutaminase in the apoptotic process is still poorly understood. One possibility is that protein cross-linking stabilizes apoptotic cells and bodies, preventing leakage of intracellular contents into the extracellular millieu (which can trigger inflammation). Alternatively, transglutaminase modification may target proteins for subsequent degradation. Intriguingly, overexpression of the enzyme has been reported to trigger apoptotic cell death [99], suggesting that transglutaminase may be a component of the death effector pathway. Further efforts are requried to identify the substrates for transglutaminase in apoptotic cells and to determine the consequences of their modification.

POSSIBLE CONSEQUENCES OF INTRACELLULAR Ca^{2+} POOL DEPLETION

In some cellular systems, extracellular or intracellular Ca^{2+} chelators actually can promote DNA fragmentation, even though other triggers of apoptosis in these systems (i.e., glucocorticoids, growth factor withdrawal) have been shown to deplete the ER Ca^{2+} store. These observations have led Baffy et al. [27] and more recently Lam and co-workers [26] to propose that depletion of the ER Ca^{2+} store may itself serve as a signal for apoptosis. How could this occur? At least two of the catabolic enzymes proposed to be involved in the effector mechanism of apoptosis (DNase I and a pool of the NS protease) are localized to the ER, and it is therefore possible that loss of Ca^{2+} leads to release of these factors into the perinuclear region or into the nuclear matrix itself. In addition, it is known that ER Ca^{2+} pool depletion results in the release of a small biomolecule that partipates in a retrograde signal for plasma membrane Ca^{2+} influx, and it is possible that it or another molecule released in a similar fashion can also promote cell death.

Depletion of mitochondrial Ca^{2+} stores may also partipate in the signal for apoptosis. Mitochondrial Ca^{2+} uptake is driven by mitochondrial membrane potential ($\Delta\Psi$) [28]. In de-energized mitochondria Ca^{2+} can be released by a reversal of the uptake pathway. Under conditions of oxidative stress, mitochondrial Ca^{2+} cycling can reach critical levels, leading to increased energy expenditure and a dramatic fall in $\Delta\Psi$. Recent work has shown that a fall in mitochondrial $\Delta\Psi$ is an early event in apoptosis [29–31], and ruthenium red, an inhibitor of the mitochondrial Ca^{2+} uptake pathway, blocks apoptosis in L929 fibroblasts [100] and inhibits the progression of apoptosis in glucocorticoid-treated splenocytes [29], suggesting that mitochondrial Ca^{2+} release is involved. Again, further efforts are required to determine the relationship between this event and the activation of the effector pathway.

ALTERNATIVE SIGNALS FOR APOPTOSIS

Oxidative stress

Several lines of evidence indicate that reactive oxygen species are involved in promoting apoptosis in diverse model systems. Treatment of cells with low to moderate doses of exogenous oxidants (i.e., hydrogen peroxide, tert-butyl peroxide, menadione) can trigger apoptosis [45, 46, 49, 68, 100–102]. Moreover, apoptosis induced by agents that are

not direct oxidants (tumor necrosis factor, glucocorticoids, thapsigargin, chemotherapeutic agents) is associated with oxygen radical production and depletion of intracelluar antioxidants (i.e., reduced glutathione) [28, 29, 46, 50, 68, 100, 103–105]. Although the identities of the oxygen radicals involved in each system are still under active investigation, roles for superoxide [30, 100], lipid peroxides [46], nitric oxide [106], and hydroxyl radicals [105] have been proposed. In these systems exogenous antioxidants such as *N*-acetyl cysteine and free radical scavengers block DNA fragmentation and cell death [50, 103–105]. Finally, several laboratories have now presented strong evidence that BCL-2 and BCL-X_L possess antioxidant properties that may be involved in their abilities to inhibit cell death. Thus, oxidative stress is another good candidate for a central cell death signal, one that may affect intracellular Ca^{2+} homeostasis. However, it should be noted that BCL-2 and BCL-X_L can still inhibit apoptosis under conditions of low oxygen [107, 108], which has been raised as an argument against a universal role for oxidative stress in the response.

Intracellular acidification

Eastman and co-workers have proposed another alternative to Ca^{2+} that may serve as a general signal for cell death. In their efforts to characterize the biochemical mechanisms underlying chemotherapy-induced apoptosis, they determined that isolated nuclei from their cellular models possessed an endonuclease activity that was stimulated by acidic pH (Dnase II) [109]. Interestingly, Collins and coworkers (personal communication) have recently identified an acidic nuclease in IL-3-dependent hematopoietic cells that can also be activated by Ca^{2+} under the appropriate conditions, suggesting that intracellular acidification and alterations in intracellular Ca^{2+} homeostasis may represent independent ways of arriving at the same endpoint (endonuclease activation) in apoptotic cells. Other efforts have shown that a drop in cytoplasmic pH precedes the morphological and biochemical features of apoptosis in certain models [110, 111], observations that have since been confirmed by other investigators in other systems [112]. Moreover, the protective effects of certain survival factors and agents that activate protein kinase C have been linked to activation of the Na^+/H^+ antiporter and intracellular alkylinization [112, 113]. Finally, the drop in pH is prevented by overexpression of BCL-2 (A. Eastman, personal communication), suggesting that acidification may represent another cellular target for this family of apoptosis suppressors. It will be interesting to determine whether intracellular acidification is related to oxidative stress and intracellular Ca^{2+} alterations in these systems.

Ceramide production

The generation of bioactive signal transduction regulators via the hydrolysis of plasma membrane phospholipids is emerging as an important general means of regulating apoptotic cell death. Of all of the second messengers shown to be involved, most recent attention has been focussed on ceramide as a possible ubiquitous trigger of apoptosis [114, 115]. Two major mechanisms have been identified that appear to contribute to the formation of ceramide under different circumstances. The most common pathway involves activation of the enzyme sphingomyelinase, which catalyzes the hydrolysis of sphingomyelin to form ceramide and diacylglycerol, a response that is involved in apoptosis induced by tumor necrosis factor, engagement of the Fas antigen, and ionizing radiation [114–120]. Ceramide can also be formed de novo via activation of ceramide synthase, and it has been shown that the cytotoxic effects of the chemotherapeutic agent daunorubicin on leukemic cell lines are mediated by this pathway [121]. Notably, exogenous hydrolysis-resistant ceramide analogues or sphingomyelinase can mimic the effects of tumor necrosis factor and the other apoptosis-inducing agents to trigger endonuclease activation and cell death, indicating that ceramide production is sufficient to induce apoptosis [115–121]. In addition, the possibility that ceramide is an evolutionary conserved trigger of apoptosis is suggested by the fact that overexpression of the *Drosophila* cell death protein, Reaper, leads to up-regulation of cellular ceramide levels (J. M. Abrams, personal communication). Interestingly, phorbol esters and diacylglycerol antagonize the death-promoting effects of ceramide in diverse models [115, 122], suggesting that a balance between ceramide and diacylglyceride may determine the outcome of death signals. The downstream targets for ceramide remain unclear, but candidates include a ceramide-dependent protein phosphatase [123], a ceramide-activated protein kinase [124], the ζ isoform of protein kinase C [125], and the H-ras protooncogene [118, J. Trent et al., unpublished results]. Whether ceramide acts upstream, downstream, or independently of the other candidate biochemical mediators of apoptosis remains to be determined.

CONCLUSIONS AND FUTURE DIRECTIONS

The independent efforts of many laboratories over the past several years have established that alterations in intracellular Ca^{2+} homeostasis are commonly involved in initiating apoptosis. Ongoing work suggests that activation of Ca^{2+}-stimulated signaling networks and catabolic enzymes represents one way these signals are translated into responses. In addition, preliminary evidence indicates that the depletion of intracellular Ca^{2+} pools can itself serve as a signal for cell death, perhaps by promoting relocalization of some of the key catabolic enzymes involved and by enhancing oxidative stress in mitochondria. In some systems other signals have been defended as key regulators of the response, and further efforts are therefore required to determine if and how all of these signals are interrelated.

An important component of the defense of Ca^{2+} as a central cell death regulator is the idea that BCL-2 and its homologues specifically regulate intracellular Ca^{2+} compartmentalization, an idea that has strong preliminary support but requires a good deal of additional investigation. In

particular, further efforts are required to determine how BCL-2 exerts its effects on Ca^{2+} and whether these effects are required for its cell death-suppressing function. Similarly, it is maintained at present that any central cell death signal would directly or indirectly activate one or more members of the ICE family of cysteine proteases, and at present there is no evidence available that any of the candidate central cell death signals are capable of this. Elucidation of these relationships over the next few years should provide new targets for therapeutic intervention that may aid in the treatment of the expanding number of diseases, including cancer, AIDS, and neurodegenerative diseases, in which apoptosis is thought to play a central role in their pathologies.

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Ion homeostasis and apoptosis

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Alterations in the transmembrane gradients of several physiological ions may influence programmed cell death. In particular, recent data suggest that increases in intracellular calcium may either promote or inhibit apoptosis, depending on the level, timing and location, whereas loss of intracellular potassium promotes apoptosis.

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Introduction

Programmed cell death leading to apoptosis is essential for normal tissue development and homeostasis, and it contributes to certain forms of pathological cell loss. Although apoptosis was originally defined by characteristic morphological features, including cell body shrinkage, nuclear condensation, chromatin margination and the formation of membrane-bound cellular remnants (apoptotic bodies), it is now better defined by its macromolecular underpinnings. Bcl-2 family proteins, including bax, bid and bcl-xL, typically regulate the release of cytochrome c and other apoptogenic factors from mitochondria; cytochrome c interacts with Apaf-1 and caspase-9 to form an 'apoptosome' capable of activating effector caspases such as caspase-3. Caspases can also be activated by cell surface 'death receptors' such as tumor necrosis factor (TNF) receptors via caspase-8 [1•].

Evidence is also emerging for a caspase-independent pathway of mammalian cell apoptosis, mediated by the mitochondrial release of apoptosis-inducing factor (AIF) [2••]. In comparison, less is understood about the accompanying changes in intracellular ionic homeostasis, although apoptotic cell shrinkage must indicate a substantial movement of water and ions across the plasma membrane. Such a shift is also central to the major alternative form of cell death, necrosis.

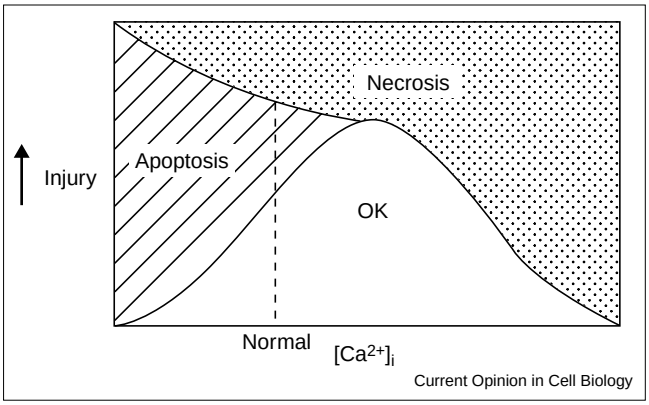
This brief review will discuss evidence suggesting that changes in intracellular ion homeostasis mediate or modify apoptosis. It will also focus on changes in intracellular free calcium ($[Ca^{2+}]_i$) and potassium ($[K^+]_i$).

Multiple roles for cellular calcium in apoptosis

Cellular Ca^{2+} overload has been suggested to be the final common pathway of cell death [3,4]. In recent years, as distinctions between necrosis and apoptosis have become better appreciated, elevated $[Ca^{2+}]_i$ has retained its status as a key factor in necrosis [5], whereas a more variegated relationship has emerged between $[Ca^{2+}]_i$ and apoptosis. As extracellular Ca^{2+} concentrations ($[Ca^{2+}]_e$) typically exceed $[Ca^{2+}]_i$ by four orders of magnitude, a large increase in Ca^{2+} influx and $[Ca^{2+}]_i$ inevitably accompanies the collapse of the plasma membrane that occurs early in necrosis. In several cases — which probably share a relatively slow rate of progression — this increase in $[Ca^{2+}]_i$ is responsible for the ensuing death. For example, membrane-toxin-induced death of hepatocytes requires extracellular Ca^{2+} [3]. Similarly, breaching the plasma membrane by sustained activation of agonist-gated Ca^{2+}-permeable channels (e.g., nicotinic acetylcholine receptors on skeletal muscle [6] or N-methyl-D-aspartate [NMDA] receptors on cortical neurons [7]) induces extracellular Ca^{2+}-dependent necrosis; in cortical neurons, average somatic $[Ca^{2+}]_i$ levels may exceed 5 μM [8]. There are many reasons why large elevations in $[Ca^{2+}]_i$ are cytotoxic, including a subsequent derangement in signaling and mitochondrial function, as well as the destruction of cellular components by Ca^{2+}-activated catabolic enzymes and free radicals. These free radicals are generated by arachidonic acid metabolism, Ca^{2+}-activated nitric oxide synthase and the uncoupling of electron transport [7,9].

Less dramatic elevations in $[Ca^{2+}]_i$ may also mediate apoptosis. Such elevations occur in synovial cells, lymphocytes, prostate cells, hepatocytes, adenocarcinoma cells and neurons undergoing apoptosis [4,10,11], both at early stages (minutes to hours after a stimulus) and late stages (post commitment point) of the apoptotic pathway. In some settings, an early increase in $[Ca^{2+}]_i$ may induce the cellular injury that then triggers apoptosis. This early Ca^{2+}-insult pattern occurs, for example, in neurons undergoing apoptosis after low-level NMDA receptor activation (see below) or in rat prostatic cancer cells undergoing apoptosis after exposure to a Ca^{2+} ionophore [12]. In other settings, an early increase in $[Ca^{2+}]_i$ may not *per se* induce cellular damage, but rather it may serve as a component of a signaling cascade culminating in triggering apoptosis. Immortalized granulosa cells subjected to serum withdrawal exhibit an almost immediate progressive increase in $[Ca^{2+}]_i$, reaching four times the resting levels within 10 minutes, and then become apoptotic over the ensuing hours; cell death can be abrogated by chelating intracellular Ca^{2+} with the calcium chelator BAPTA [13]. A similar moderate early increase in $[Ca^{2+}]_i$ has been observed in PC12 cells [14] and cochlear neurons [15] exposed to the broad-spectrum kinase inhibitor, staurosporine, and in thymocytes exposed to thapsigargin (see below), suggesting that moderate increases in $[Ca^{2+}]_i$ might be a common mechanism.

Figure 1



A speculative diagram illustrating a relationship between an apoptosis–necrosis continuum and two key variables, insult severity (injury) and intracellular free Ca^{2+} levels. High intracellular Ca^{2+} levels or more severe insults promote cell death through necrosis, whereas low intracellular Ca^{2+} levels or milder insults promote cell death through apoptosis. Lowering Ca^{2+} may induce apoptosis, even in relatively healthy cells, whereas modestly raising Ca^{2+} may inhibit apoptosis. Reprinted from [5].

Late elevations in $[Ca^{2+}]_i$ have been implicated in the execution of apoptosis. Tombal *et al.* [16••] recently used microinjected fura dextran to follow $[Ca^{2+}]_i$ longitudinally in prostate cancer cells undergoing apoptosis after exposure to thapsigargin, and they observed both an early (0–12 hr) hundred nM rise, and a second, late (12–96 hr) rise in $[Ca^{2+}]_i$. Induction of Jurkat cell apoptosis via exposure to anti-Fas antibody resulted in a delayed increase in $[Ca^{2+}]_i$ but the increase persisted until membrane failure; addition of BAPTA and removal of extracellular Ca^{2+} inhibited this increase in $[Ca^{2+}]_i$ as well as DNA fragmentation but not cell shrinkage or several other apoptotic events [17]. A late elevation in $[Ca^{2+}]_i$ was also observed in thymocytes [18]. Possible targets of a late rise in $[Ca^{2+}]_i$ during apoptosis include key Ca^{2+}-activated proteases [19] and endonucleases, such as NUC18 [20,21], as well as perhaps caspase-3 [22•].

What determines whether an increase in $[Ca^{2+}]_i$ leads to apoptosis or necrosis? The magnitude of the increase may be a critical factor. Apoptosis has been considered a default, 'altruistic' death pathway potentially resulting from any insult; in this view, necrosis occurs only if time is too short (i.e., insult severity is too great) to permit such an orderly death [23]. Typically, early increases in $[Ca^{2+}]_i$ in cells undergoing apoptosis are only several-fold higher than resting levels (see above). In cortical neurons, where either apoptosis or necrosis may occur after NMDA-receptor-induced $[Ca^{2+}]_i$ elevation, apoptosis is favored by milder insults and preservation of mitochondrial energy production [24]; these cells may have an intrinsic cellular propensity to undergo apoptosis, which may be influenced by factors such as maturity [25]. In addition, the source or subcellular localization of increased $[Ca^{2+}]_i$ probably influences its effect on apoptotic pathways.

Several studies have suggested that Ca^{2+} release from the endoplasmic reticulum and/or capacitative Ca^{2+} influx through Ca^{2+} release-activated Ca^{2+} channels (I_{CRAC}) are apoptogenic [26,27]. Ca^{2+} released from cellular stores may trigger apoptosis owing to their proximity to mitochondria (mitochondrial Ca^{2+} uptake and consequent overload may result in transition pore opening and cytochrome c release [28•]). In addition, Ca^{2+} depletion from the endoplasmic reticulum may impair protein synthesis ([29]; see also below). It has been proposed that bcl-2 inhibits Ca^{2+} release from the endoplasmic reticulum [30], although this may not be the case with bcl-xL [31].

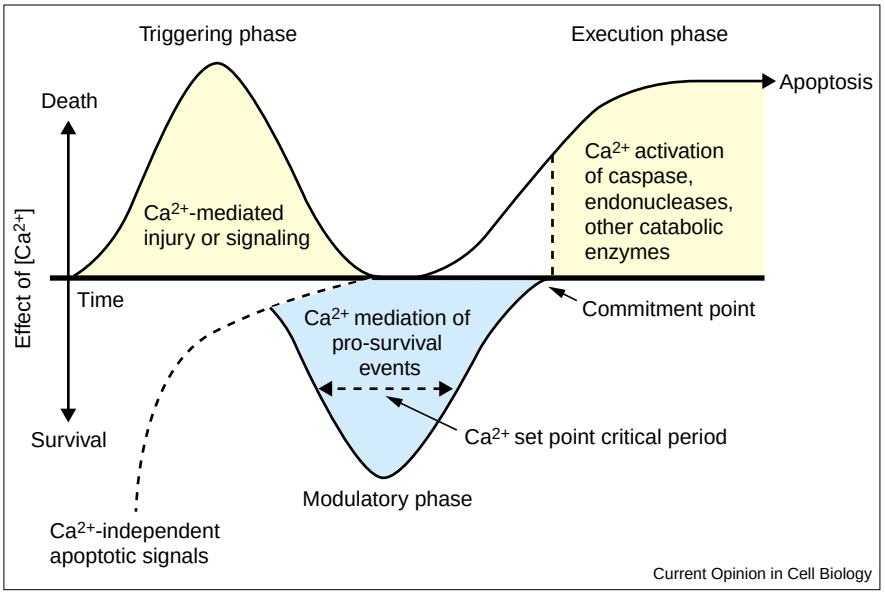
Even without considering subcellular localization, the links between elevated $[Ca^{2+}]_i$, at least (as usually measured) when averaged over the cell body, and apoptosis are not straightforward. First, not all dying cells exhibit elevations in $[Ca^{2+}]_i$ [32] prior to final membrane failure [33]; indeed, reduced $[Ca^{2+}]_i$ has been observed in apoptotic myeloid cells [34] and neurons [35,36]. Second, lowering extracellular $[Ca^{2+}]$ or blocking membrane Ca^{2+} channels can itself trigger apoptosis *in vitro* [37] or enhance pathological neuronal apoptosis *in vivo* ([38••]; Figure 1). And third, modestly elevating $[Ca^{2+}]_i$ towards an optimal 'set-point' can inhibit apoptosis ([39]; Figure 1). Extracellular K^+-induced activation of voltage-gated Ca^{2+} channels improves the survival of sympathetic neurons deprived of nerve growth factor [39] (an additional effect of limiting K^+ efflux is mentioned below). Similarly, developmental [37,40] or insult-induced [36] neuronal apoptosis is reduced by enhancing calcium influx. Treatment with a calcium ionophore or promoting calcium influx via magnetic fields inhibits apoptosis in myeloid leukemic cells [41,42].

Further studies are needed to determine why raising $[Ca^{2+}]_i$ can inhibit apoptosis. $[Ca^{2+}]_i$ mediates multiple signaling cascades that are critical for cell survival [43], including Ca^{2+}/calmodulin-dependent protein kinase kinase, protein kinase B and the phosphorylation of BAD [44]. As noted above, loss of Ca^{2+} from endoplasmic reticulum stores may be especially important in inducing apoptosis (see also [31,45,46••]), suggesting that a key survival-promoting effect of raising $[Ca^{2+}]_i$ may be the maintenance of these stores (see Figure 2).

Potassium efflux and apoptosis

As K^+ is the predominant intracellular cation (typically present at around 140 mM), it is plausible that apoptotic cell shrinkage would be accompanied by loss of cellular K^+ (Figure 3). Barbiero *et al.* [47] used the K^+-sensitive fluorescent dye PBFI to estimate that the $[K^+]_i$ in apoptotic mouse L fibroblast cells falls below 50 mM. This would also suggest that K^+ efflux exceeded water efflux. Measurements performed on CEM-C7A lymphoma cells during dexamethasone-induced apoptosis also confirmed early K^+ loss [48], which may amount to 50–75% of the initial $[K^+]$ [49,50]. Other early clues pointing to a role for K^+ efflux in apoptosis were provided by demonstrations that

Figure 2



Bidirectional regulation of apoptosis by increased $[Ca^{2+}]_i$. A speculative diagram illustrating three phases in the relationship between elevations in $[Ca^{2+}]_i$ and apoptosis. In the first, triggering phase, modest elevation in $[Ca^{2+}]_i$ either induces critical levels of cellular injury or serves as an early signaling mediator of the apoptosis cascade. Apoptosis can also be triggered by events independent of changes in $[Ca^{2+}]_i$. In the second, modulatory phase, cells exhibit relative Ca^{2+} starvation; average cytosolic $[Ca^{2+}]_i$ may even be subnormal. Modest elevations in $[Ca^{2+}]_i$ during this critical period may inhibit apoptosis, perhaps by restoring signaling homeostasis and endoplasmic reticular function. In the third, execution phase, after cells are committed to die, a large increase in $[Ca^{2+}]_i$ mediates final events such as the activation of proteases and endonucleases. In this timing diagram, K^+ loss may primarily occur during the middle, modulatory phase (see [58]).

the K^+ ionophore valinomycin induced apoptosis [51] (although valinomycin also disturbs mitochondrial function [52]) and that K^+ channel blockers reduced apoptotic cell shrinkage in eosinophils [53] or HL-60 cells [49].

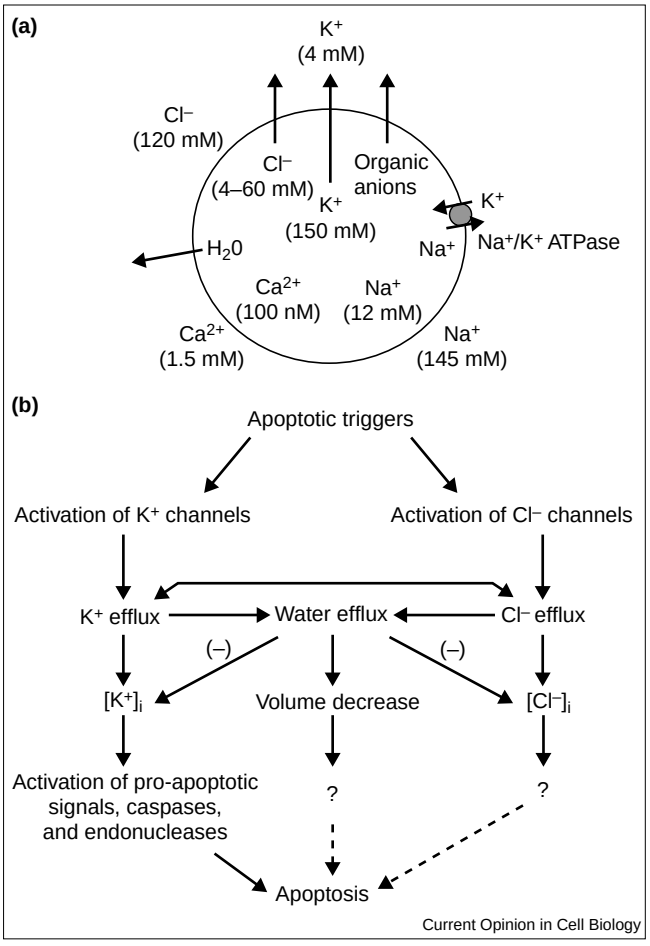
In the past few years, evidence has accumulated suggesting that K^+ efflux leading to a decrease in $[K^+]_i$ may be a critical driver of apoptosis. Electrophysiological examination of cortical neurons undergoing apoptosis in several settings revealed early enhancement of the delayed rectifier K^+ current, I_K, leading to net cellular K^+ loss [54,55]. Blocking K^+ current with the K^+ channel blocker tetraethylammonium (TEA) or by elevating extracellular K^+ inhibited apoptosis, even though secondary increases in $[Ca^{2+}]_i$ were prevented. Concurrently, Cidlowski and colleagues [56,57] estimated that $[K^+]_i$ fell to 35–50 mM in lymphocytes undergoing apoptosis and that apoptosis was inhibited when K^+ efflux was blocked by raising extracellular K^+. Cidlowski and colleagues [56,57] also determined that the proteolytic activation of caspase-3 and activation of nuclease in solution was facilitated by comparable reduction in $[K^+]$, with half-inhibition $\cong$ 40 or 80 mM, respectively [56]. Similarly, K^+ inhibited the ability of mitochondrial supernatants to induce chromatin condensation and DNA fragmentation in isolated HeLa nuclei [58]. An early, TEA-sensitive increase in extracellular K^+ was recently measured in the vicinity of mouse zygotes undergoing enhanced apoptosis *in vitro* [59]; high K^+ medium prevented caspase activation and internucleosomal DNA fragmentation in ovarian granulosa cells exposed to doxorubicin [60].

There is also recent evidence that decreased $[K^+]_i$ may facilitate early apoptotic signaling. In myeloblastic leukemia cells exposed to UV irradiation, the K^+ channel blocker 4-aminopyridine (4-AP) reduced activation of the JNK/SAPK signaling pathway and subsequent apoptosis [61]; in HeLa, U937 and PC12 cells, K^+ channel blockers reduced staurosporine-induced cytochrome c release and apoptosis [62••]. Expression of the voltage-gated Kv1.3 channel broadly reduces protein tyrosine phosphorylation in HEK293 cells [63], a pro-apoptotic event (see for example: [64]).

Given the more than 200 different K^+ channel genes with two, four or six putative transmembrane segments that have now been identified [65], one might anticipate that different K^+ channels might mediate apoptotic K^+ efflux in different cell types. So far, it appears that apoptotic K^+ efflux is typically mediated by high conductance, non-inactivating voltage- or Ca^{2+}-dependent channels such as delayed rectifier I_K channels [54,61,66,67], Ca^{2+}-dependent maxi-K^+ channels [68,69] or inward rectifying K^+ channels [70••]. Upmodulation of I_K-like channels during apoptosis has been observed in cholinergic septal cell lines [66], myeloblastic leukemia cells [61], liver cells [71] and rat vascular smooth muscle cells [69]. Reduced outward K^+ current has been proposed to underlie the anti-apoptotic effects of infectious bursal disease viral infection upon chicken fibroblasts [68] or TNF-α upon retinal ganglion cells [67]. Expressing Kir1.1 inward rectifier K^+ channels induced apoptosis in rat hippocampal neurons [70••], and expressing Kv2.1 delayed rectifier K^+ channels in HEK 293 cells enhanced susceptibility to ceramide-induced apoptosis (SP Yu, DW Choi, unpublished data). K^+ channel blockers demonstrated to inhibit apoptosis, besides the drugs already mentioned, include tetrapentylammonium (TPeA), tetrahexylammonium (THA), spateine, quinine, quinidine, clofilium and the maxi-K^+ channel blocker, iberiotoxin [49,55,69,72,73].

Besides voltage- or Ca^{2+}-dependent K^+ channels, a possible role for agonist-gated channels, specifically NMDA

Figure 3



Apoptotic volume decreases and possible links to apoptosis. (a) K^+ efflux, accompanied by the efflux of $Cl^–$, organic anions and water, is a plausible prerequisite for cellular volume reduction. The Na^+/K^+-ATPase and other ion transporters and exchangers probably also participate in cell volume control and ion homeostasis. Typical ion concentrations are depicted; however, these vary somewhat in different cell types. (b) A speculative model outlining the possible participation of three highly interlinked events in apoptotic shrinkage. Double lines depict inhibitory events and dotted lines depict undefined relationships. Apoptotic triggers may activate K^+ or $Cl^–$ channels, leading to K^+ and Cl efflux, water efflux, and cell body shrinkage. K^+ efflux and $Cl^–$ efflux are mutually reinforcing, due to charge effects. If K^+ or $Cl^–$ efflux occurs to a greater extent than water efflux, $[K^+]_i$ or $[Cl^–]_i$ will fall. Decreases in $[K^+]_i$, $[Cl^–]$ or volume may each independently promote apoptosis; alternatively one event may critically promote apoptosis and the effects of other events may be primarily mediated through that event. Modified from Figure 1 in [81] with permission.

receptor-gated channels (which are highly permeable to K^+), has been identified in central neuronal apoptosis [74]. K^+ loss mediated by reduced membrane Na^+/K^+-ATPase activity may contribute to CD95-induced apoptosis in Jurkat cells [75]. However, not all studies are consistent with the hypothesis that K^+ efflux promotes apoptosis. For example, the K^+ channel opener cromakalim prevented apoptosis in hippocampal neurons [76]; the anti-apoptotic effect of expressing a bcl-2 family gene, *mcl-1*, in myeloblastic cells was associated with enhanced opening of a 4-AP-sensitive K^+ channel [77•]; and 4-AP or clofilium alone can induce apoptosis [78,79]. In these settings, it is possible that experimentally induced modulation of K^+ efflux critically altered membrane potential (and thus had a larger, opposite effect upon Ca^{2+}-dependent or depolarization-activated K^+ channels) or that the drugs employed had other actions.

Assuming that further studies indeed support a general role for K^+ channel upregulation or upexpression in apoptotic cascades, central questions for the future study will be the identity of the specific K^+ channels involved and of the signaling pathways responsible for K^+ channel enhancement, which may be mediated by channel protein phosphorylation. In Jurkat cells, Fas-induced K^+ loss, cell shrinkage and apoptosis were blocked by PKC stimulation and enhanced by PKC inhibition [80]. In cortical neurons, inhibition of tyrosine kinases with herbimycin A or lavendustin suppressed the enhancement of I_K and apoptosis induced by ceramide [55].

Chloride channels, volume control and apoptosis

Two events closely associated with intracellular K^+ and $Cl^–$ efflux and water efflux/cell shrinkage have also been implicated in the regulation of apoptosis, although it is presently unclear to what extent the influence of these is mediated independently, rather than through secondary effects on K^+ efflux and $[K^+]_i$ ([81]; Figure 3). An outwardly rectifying $Cl^–$ channel is activated in Jurkat T lymphocytes by apoptotic signals [82], and elevating extracellular $Cl^–$ or adding pharmacological $Cl^–$ channel blockers can inhibit DNA fragmentation [83] and apoptosis [62••,82]. Unfortunately, presently available pharmacological blockers for $Cl^–$ channels are poor. Cell volume reduction may play a mechanistic role in apoptosis, and hypertonic medium induce or potentiate apoptosis in some studies (for example, see [84]), and regulatory volume decrease responses are enhanced in HeLa, U937, PC12 and NG108-15 cells undergoing apoptosis [62••]. However, volume control mechanisms were not altered in CEM-C7A cells undergoing apoptosis after exposure to dexamethasone [48], and modification of apoptosis by non-specific effects of osmotic stress is difficult to exclude.

Other ions

A role for excess Mg^{2+} entry and toxic elevations of intracellular Mg^{2+} levels in glutamate-induced neuronal death has been proposed [85]. The toxic translocation of endogenous Zn^{2+} from presynaptic nerve terminals into the cytosol of postsynaptic target neurons has been implicated in the pathogenesis of selective neuronal death after global ischemia [86,87], which is probably, at least in part, apoptotic [11]. The surprising finding of elevated Zn^{2+} levels in neurons dying after sustained seizures in mutant mice lacking presynaptic Zn^{2+} stores [88] has raised the additional possibility that toxic Zn^{2+} may originate from internal cellular stores [89]. Altered copper homeostasis

may induce cytotoxicity, perhaps apoptosis, in certain disease states such as Wilson's disease or familial amyotrophic lateral sclerosis (ALS) [90]. Finally, mobilization of endogenous intracellular iron may participate in apoptosis, perhaps by facilitating hydroxyl radical formation. The anti-apoptotic effect of the stress-induced protein, haem oxygenase-1, is associated with increased iron efflux and iron chelators inhibit apoptosis [91].

Conclusions

Alterations in the cellular homeostasis of several physiological ions appear to be important modifiers or mediators of the apoptotic cascade. Although Ca^{2+} or K^+ have figured most prominently in studies to date, pro-apoptotic effects of Cl^- and water efflux have been proposed, and other ions may participate in certain circumstances. Further examination of the relationship between ionic homeostasis and apoptosis is warranted and may aid identification of strategies for the therapeutic manipulation of apoptosis in disease states.

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Characterization of Calcium Release-activated Apoptosis of LNCaP Prostate Cancer Cells*

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Apoptosis inhibition rather than enhanced cellular proliferation occurs in prostate cancer (CaP), the most commonly diagnosed malignancy in American men. Therefore, it is important to characterize residual apoptotic pathways in CaP cells. When intracellular Ca^{2+} stores are released and plasma membrane "store-operated" Ca^{2+} entry channels subsequently open, cytosolic [Ca^{2+}] increases and is thought to induce apoptosis. However, cells incapable of releasing Ca^{2+} stores are resistant to apoptotic stimuli, indicating that Ca^{2+} store release is also important. We investigated whether release of intracellular Ca^{2+} stores is sufficient to induce apoptosis of the CaP cell line LNCaP. We developed a method to release stored Ca^{2+} without elevating cytosolic [Ca^{2+}]; this stimulus induced LNCaP cell apoptosis. We compared the apoptotic pathways activated by intracellular Ca^{2+} store release with the dual insults of store release and cytosolic [Ca^{2+}] elevation. Earlier processing of caspases-3 and -7 occurred when intracellular store release was the sole Ca^{2+} perturbation. Apoptosis was attenuated in both conditions in stable transfected cells expressing antiapoptotic proteins $Bclx_L$ and catalytically inactive caspase-9, and in both scenarios inactive caspase-9 became complexed with caspase-7. Thus, intracellular Ca^{2+} store release initiates an apoptotic pathway similar to that elicited by the dual stimuli of cytosolic [Ca^{2+}] elevation and intracellular store release.

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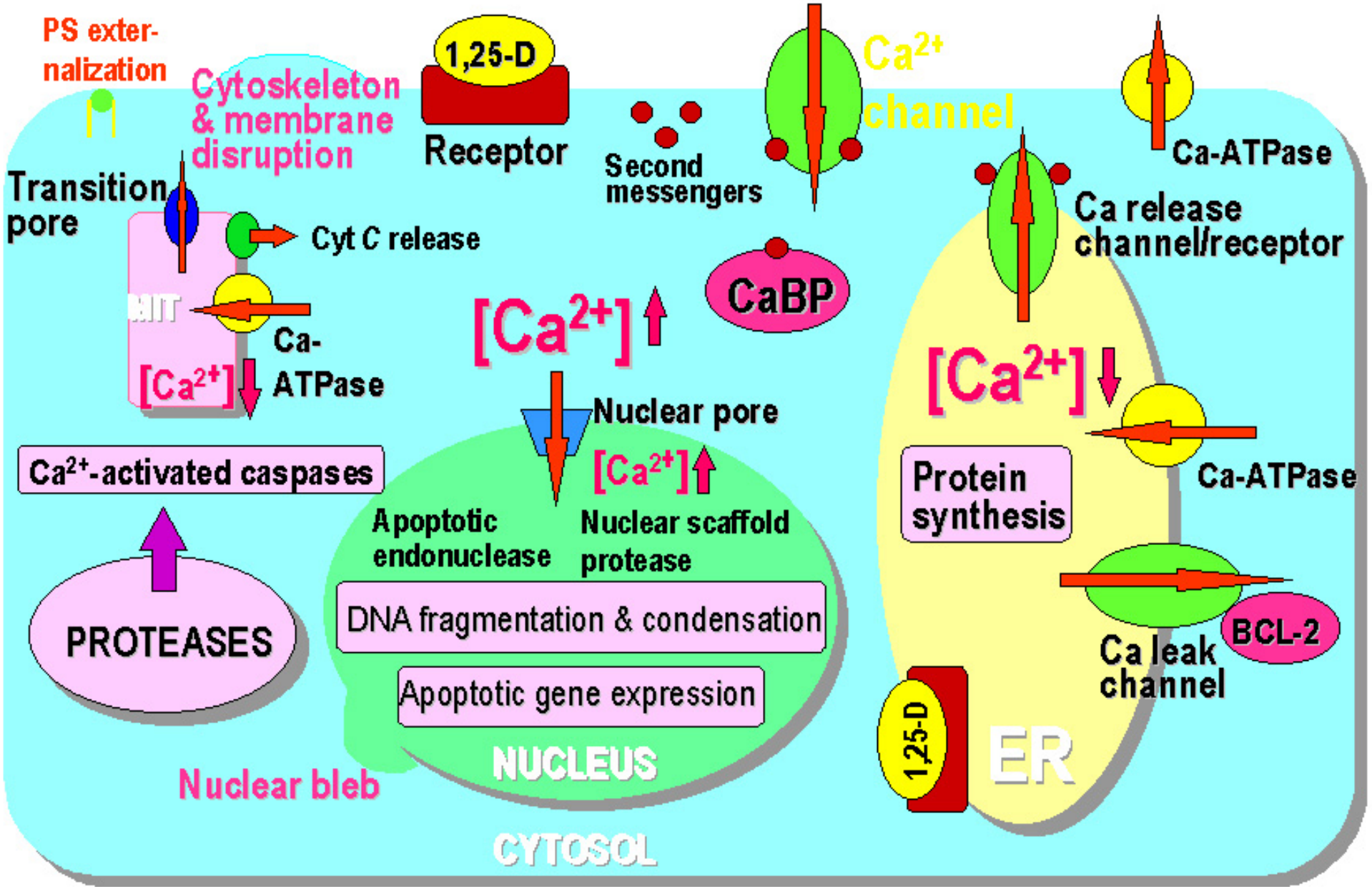
Intracellular Ca^{2+} and Apoptosis



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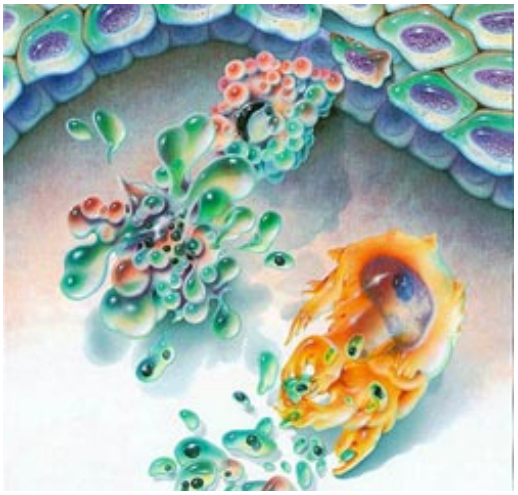
Nanoprobes light up cellular demolition

06 March 2006

South Korean researchers have developed a method for detecting and imaging programmed cell death (apoptosis). The method, involving polymer nanoparticles, could be used to diagnose apoptosis-related diseases and inform drug development, say the researchers.



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Apoptosis is an essential process in the development and maintenance of all multi-cellular organisms. About 50-70 billion cells are killed each day by apoptosis in the average human adult. But when the process goes wrong, when cells fail to die, for example, then diseases such as cancer can result.

Current apoptosis probes are based on the fluorescently-labelled protein annexin V, which binds to a special phospholipid exposed on the surface of apoptotic cells. But this phospholipid can sometimes appear on the surface of healthy cells.

Researchers led by Ick Chan Kwon from the Korea Institute of Science and Technology, Seoul, developed a probe for detecting two cysteine proteases (caspases) that play an integral role in the apoptosis process. Caspases effectively take cells to bits. The researchers linked the specific amino acid sequence that is cleaved by the caspases to a fluorescent compound, and then attached this to biocompatible polymer nanoparticles made of poly(ethyleneimine).

Chan Kwon's team showed that these nanoparticles entered healthy cells without damaging them, and did not fluoresce at first. The nanoparticles only started fluorescing when the researchers instigated apoptosis by treating the cells with tumour necrosis factor. At this point the nanoparticles interacted with the two specific caspases.

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ELSEVIER FULL-TEXT ARTICLE

Pro-oxidants and mitochondrial Ca2+: their relationship to apoptosis and oncogenesis.

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Apoptosis is a physiological process for active cell removal. One of its hallmarks is an increased cytosolic Ca2+ content. Several genes involved in apoptosis control have been identified, but their mode of action is not understood in detail. Apoptosis may relate to oncogenesis, in that some malignant tumors may grow because genes engaged in apoptosis control are altered. L929 cells overexpressing the proto-oncogene bcl-2 have an increased mitochondrial membrane potential (delta psi), as have many carcinoma cells. bcl-2 protects L929 cells against apoptosis caused by pro-oxidant-induced mitochondrial Ca2+ 'cycling' and increased cytosolic Ca2+ levels. Nerve growth factor, which induces catalase, and inhibitors of mitochondrial Ca2+ release also prevent apoptosis. It is suggested that a pro-oxidant-induced Ca2+ release from mitochondria, followed by Ca2+ cycling and ATP depletion, is a common basic event during apoptosis. Accordingly, maintenance of delta psi stabilizes mitochondria, thereby prevents apoptosis, and may confer increased growth potential to cells.

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Vitamin D and Breast Cancer

Breast cancer is the most common malignancy of women in the western world. Many factors contribute to causing breast malignancy (it is multifactorial) with heredity a major factor. Certain diets also help prevent it, such as diets high in vegetables and fruit and low in fat. Adequate calcium is very important. The role of vitamin D in both the prevention and treatment of breast malignancy is being intensively explored by scientists but is still preliminary.

No matter what cancer you have, or are trying to prevent, the real question is should cancer patients be left vitamin D deficient? Many experts will tell you that vitamin D should not be taken for breast cancer until well controlled scientific studies prove it helps. The problem with that approach is two-fold. First, you may die waiting for the studies to be conducted and two, it misses the point. The point is this: women with breast cancer should not allow themselves to be vitamin D deficient and neither should their doctors.

If you have breast cancer, please remember that vitamin D is not a cure-all and should never be used as the main treatment for your cancer. Your oncologist will prescribe treatment that has proven efficacy and you should carefully follow his/her advice as the mainstay of treatment. At the same time, you should know that evidence suggests that the proper amount of vitamin D may help you in your fight against breast cancer.

Next, let's look at selected studies from the scientific literature to see what clues exist about the role vitamin D may play in preventing, and treating, breast cancer.

In 1989, the prestigious medical journal, The Lancet, reported that the most active form of vitamin D (calcitriol or 1,25-OH-D) significantly reduced the growth of breast cancer in an animal model. Furthermore the researchers (from St. George¹s Hospital medical School in London) found women who had vitamin D receptor positive tumors had longer disease free intervals than women whose tumors had no measurable receptors for vitamin D.
Lancet. 1989 Jan 28;1(8631):188-91.

Current research suggests most, if not all, women would have those vitamin D receptors unless they were deficient in vitamin D, that is, they would have those receptors if they were vitamin D replete. It seems as if the receptor is present in breast tissue if the most active form of vitamin D has been present and that is only the case if the less-active form [the substrate, calcidiol or 25 (OH)D] has been present. In other words, if you test vitamin D deficient breast cancer patients for vitamin D receptors, they will not have many; if you treat their deficiency, they will probably develop those receptors.

Not only does the active form of vitamin D, calcitriol (the form made in optimal quantities by your body when your vitamin D blood levels are ideal) inhibit breast cancer cells from growing, it makes those cells grow and die more like natural cells. Furthermore, vitamin D inhibits the formation of excessive blood vessel growth around the cancerous tumor, a process called anti-angiogenesis.
Braz J Med Biol Res. 2002 Jan;35(1):1-9.

In the 1990's, a group of scientists from the University of California at San Diego provided the first look at how many women may be dying needlessly from breast cancer due to low vitamin D blood levels. The researchers measured the amount of sunlight available to the women at the latitude where they lived and combined that with the frequency of cloudy weather. Sunny climates are associated with higher vitamin D levels. They found that women in the sunniest

regions of the USA were about half as likely to die from breast cancer as were women who lived in less sunny regions. When the same researchers looked at the USSR, before that country dissolved, they found that women who lived in the sunniest regions were three times less likely to develop breast cancer than were the women who lived in regions without as much sun.
Prev Med. 1990 Nov;19(6):614-22.
Int J Epidemiol. 1990 Dec;19(4):820-4.

In 1994, a researcher at the Memorial Sloan-Kettering Cancer Center reviewed the literature up to that date and concluded that higher intakes of vitamin D and calcium might reduce breast cancer by protecting against the carcinogenic effects of a high fat diet. He also pointed out the vitamin D intakes were far below the government recommendations in force at the time.
Adv Exp Med Biol. 1994;364:109-14.

In 1997, researchers at the Manchester Royal Infirmary discovered that women with the highest levels of activated vitamin D (calcitriol) in their blood had the best prognosis. Those women with the lowest levels had a more rapidly fatal course. They also found that women with breast cancer had low levels of 25(OH)D (calcidiol) in their blood with average levels of about 16 ng/ml. Women who live in sunny climates, where breast cancer is more rare, frequently have blood levels three times higher.
J Clin Endocrinol Metab. 1997 Jan;82(1):118-22.

However, studies that measure the blood level of activated vitamin D, calcitriol [1,25(OH)D] miss the important fact that blood levels do not reflect tissue levels. In fact, blood levels of calcitriol are quite different than tissue levels which can not be measured. However, tissue levels can be estimated from calcidiol levels as calcidiol is converted into calcitriol in the tissues and that conversion is directly proportional to the blood level of calcidiol. Simply put, that means the higher your blood levels of calcidiol, the higher the tissue levels of calcitriol and the more activated vitamin D one has in her tissues to fight breast cancer.

In 1999, researchers at the University of North Carolina School of Medicine reported that white women with the lowest blood levels of calcitriol [1,25(OH)D] were five times as likely to develop breast cancer as were women with the highest levels but the relationships did not hold for black women. More importantly, the researchers found that women with breast cancer had very low levels of 25(OH)D in their blood with average levels of 15 ng/ml for white women while the level was only 8.9 ng/ml for black women, which is severely deficient. This extraordinarily low level of calcidiol in Blacks probably explained the researchers finding about calcitriol. Blacks were so deficient in vitamin D that their kidneys could not make enough calcitriol to compensate for their low calcidiol levels. Remember, as vitamin D deficiency worsens, the kidney activates more and more calcidiol into calcitriol to maintain serum calcium leaving very little left over for the tissues to fight cancer.
Public Health Nutr. 1999 Sep;2(3):283-91.

Also in 1999, researchers the Northern California Cancer Center and the University of Miami, followed 5009 women for 20 years, as part of a large NHANES I study. 190 of the women subsequently developed breast cancer. The researchers did not have 25(OH)D (calcidiol) blood levels available, so they looked at many markers of vitamin D levels, such as living in sunny climates, sun damaged skin (indicted past sun exposure), a history of occupational and recreational sun exposure and dietary vitamin D.

All of these factors reduced the risk of breast cancer. Dietary vitamin D reduced the risk a little (due to the tiny doses of vitamin D consumed) but women with high occupational and recreational sun exposure who lived in a sunny climate reduced their risk three fold. Remember, 90 % of our vitamin D comes from sun exposure. Vitamin D from diet and supplements is close to insignificant due to the small amounts consumed.
Cancer Epidemiol Biomarkers Prev. 1999 May;8(5):399-406.

In 2002, researchers at St. George's Hospital Medical School in London reviewed the multiple mechanisms by which activated vitamin D [calcitriol or 1,25 (OH)D] prevents breast cancer. calcitriol arrests the aberrant progression of breast cancer by regulating cell cycles, forcing apoptosis (cell death), resisting signals from substances that cause cancer cells to grow,

inhibiting invasion into normal tissue and preventing metastasis. All in all, calcitriol, the most potent form of vitamin D, appears to be the perfect chemotherapeutic agent to both prevent and treat breast cancer. Unfortunately, the researchers appeared to be unaware that the best way to elevate tissue levels of calcitriol is to elevate blood calcidiol levels and the best way to elevate calcidiol levels is to take physiological doses of cholecalciferol.
Endocr Relat Cancer. 2002 Mar;9(1):45-59.

Instead of giving simple cholecalciferol to patients with breast cancer, the medical-industrial complex continued to test the potentially profitable vitamin D analogs which are patenable variations of calcitriol. The vitamin D analogs are chemical modifications of calcitriol which try to retain calcitriol's ability to fight breast cancer while not causing the high blood calcium that calcitriol usually causes. Many different vitamin D analogs were tested and many worked great in the test tube. However, just like calcitriol, they usually caused high blood calcium when given to humans.

No one seemed to care that optimal doses of cholecalciferol would raise tissue levels of cancer fighting calcitriol quite high, would not cause hypercalcemia and should work well against breast cancer. Remember, cholecalciferol occurs naturally, can not be patented and is dirt cheap. Therefore, the idea it could help breast cancer offered no financial incentives to drug companies or researchers hoping to discover a drug they could patent. Also, few of the scientists working to cure cancer had any but the most rudimentary understanding of basic vitamin D physiology, pharmacology or toxicology.
Recent Results Cancer Res. 2003;164:333-48.

Then, things started coming to a head in the last few years. In 2004, a group at the University Hospital in Quebec confirmed that vitamin D, especially when taken with calcium, significantly reduced abnormal mammograms. In fact they found women with the highest vitamin D intake had only one fourth as may abnormal densities on their mammogram as did women with the lowest intake.
Cancer Epidemiol Biomarkers Prev. 2004 Sep;13(9):1466-72.

Researchers in Germany then tested fresh breast cancer cells to see if they could activate vitamin D. Up until then, only breast cancer cells grown in test tubes had been tested. The researchers found fresh breast cancer cells could indeed activate vitamin D. Indeed those cells seemed to be hungry for the vitamin D as the cells showed increased production of the enzymes necessary to activate vitamin D. It seemed all that was missing was the vitamin D.
Recent Results Cancer Res. 2003;164:239-46.

Then researchers in Norway discovered that women who were diagnosed with breast cancer during the summer and fall, the season where vitamin D levels are the highest, had the best prognosis. The researchers concluded that high vitamin D levels during the course of cancer treatment may improve the prognosis of women with breast cancer. Colon and prostate cancer showed similar improvements.
Cancer Causes Control. 2004 Mar;15(2):149-58.

What does all this mean? To date, no studies have been published correlating vitamin D blood levels with the progression of breast cancer although such studies have been done for prostate cancer. We know that most women with breast cancer are vitamin D deficient because most women in the industrialized world are vitamin D deficient. Furthermore, women with breast cancer tend to be older, chronically ill and are often Black, all risk factors for severe vitamin D deficiency.

Of course, many questions are unanswered. However, many questions are always unanswered, that is the nature of science. It certainly looks as if vitamin D supplementation should help reduce the rate of the growth of breast cancer but that is not proven.

Although no studies prove that vitamin D helps breast cancer, no studies have ever been done to answer that simple question. Hundreds of thousands of women will die this year from breast cancer and most will be vitamin D deficient.

If asked, most scientists will tell you that vitamin D should not be given to breast cancer patients until vitamin D is proved to be both safe and effective. However, that is not the question.

The question is, should breast cancer patients be allowed to die from their cancer while not being treated for their vitamin D deficiency. We don¹t think so, and neither would most victims.

Remember, vitamin D is always an ancillary treatment, never the primary treatment. However, to battle breast cancer with low vitamin D levels makes no sense and will likely reduce your risk of surviving the disease.

So the question is, what can you do now, based on what is known now. Say you cannot wait for science? The Vitamin D Council will not tell you what to do. We are a non-profit educational organization but we are not your doctors. We will not make any recommendations. In the future, we plan to publish an e-book that will tell you what we would do if we developed breast cancer.

Department of Biological Sciences

Pathways

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COLLEGE OF SCIENCE UNIVERSITY OF NOTRE DAME

breast cancer research: the promise of VITAMIN D

JoEllen Welsh, Professor of Biological Sciences

Before she steps up to the microphone, **JoEllen Welsh** knows well in advance that her audience of breast cancer survivors will subdivide her every word in search of any hint of news on which to pin their hopes.

Carefully, Welsh will describe her research on the active form of Vitamin D, specifically 1,25-dihydroxycholecalciferol in connection with Notre Dame's Cellular and Physiological Dynamics (CPD) Cluster in the Department of Biological Sciences.

Vitamin D does more than just build strong bones and teeth. The evidence is increasingly clear that Vitamin D plays a yet-to-be-understood role in inhibiting the proliferation of cancer cells.

This mysterious role teases researchers like Welsh and cancer victims who are intrigued by Vitamin D's promises.

"On the one hand you want to give them hope. And yet we don't have the kind of breakthrough in cancer research that is ready for prime time," Welsh said. Many of the women in the audience have more than themselves in mind. "They are concerned because they have daughters who are at risk," she continued.

Science journals offer convincing evidence that Vitamin D plays a role in reducing the threat of colon and prostate cancer. When Welsh addresses local cancer survivor groups, she goes to great lengths to describe what goes on in her laboratory at the Galvin Life Science Building.

"They are very interested in how we study cancer. They want to know, for example, how we induce cancer in mice, how we manipulate and measure the tumors," she related. The give-and-take between her and the members of her audience is often conducted at a scientifically high level because "these people have been living with the disease and they are very educated about cancer."

Vitamin D is not a vitamin at all. It is an essential nutrient that is naturally manufactured in response to sunlight from cholesterol in our skin. It is a precursor to a steroid hormone that interacts with a particular receptor—the Vitamin D receptor (VDR)—to regulate calcium and bone homeostasis, cell proliferation, cell signaling, and immune function. It is not present in many foods, the major exceptions being liver and fatty fish.

Cod liver oil is naturally loaded with Vitamin D. Milk is fortified with it. And then there are over-the-counter vitamin supplements, which Welsh recommends people in northern climes take in moderation, especially during winter. Vitamin D can be stored for months in our fat, muscle, and liver. But by December and January those levels begin to drop.

Welsh works specifically in the Department of Biological Sciences on Vitamin D and its mechanism within mammary glands. In mammary tissue, Vitamin D maintains the growth of the cells that produce milk.

Vitamin D actions in breast cancer cells

Nuclear receptors are ligand-dependent transcription factors that regulate cell proliferation, differentiation, and apoptosis via changes in gene transcription. The nuclear receptors under study in the Welsh lab include the Vitamin D receptor (VDR), the ligand of which is 1,25-dihydroxycholecalciferol [1,25D], the active form of Vitamin D; and the estrogen receptor (ER), the ligands of which include estrogen, anti-estrogens, and phyto-estrogens. The lab studies mechanisms by which these nuclear receptors and their diverse ligands interact in growth regulation of normal and transformed mammary epithelial cells. Studies have shown that activation of ER stimulates, whereas activation of VDR inhibits, growth of breast cancer cells, and studies to define the mechanisms of these opposing effects are a major focus in Welsh's lab. Her lab was the first to report that 1,25D can induce apoptosis, or programmed cell death, in breast cancer cells, and has found that 1,25D-mediated apoptosis is mechanistically distinct from apoptosis triggered by cell surface death receptors that activate caspase-dependent pathways. These findings suggest that cancers that are resistant to chemotherapeutic drugs may be responsive to Vitamin D-based therapeutics. Indeed, numerous drug companies are developing synthetic Vitamin D analogs for various disorders, including those characterized by abnormal cell growth such as psoriasis, leukemias, and solid cancers. In preclinical studies with animal models, Welsh's lab has demonstrated that these Vitamin D analogs can induce regression of breast tumors via induction of growth arrest and apoptosis. Their studies have shown that Vitamin D analog therapy is effective in both estrogen-dependent, early-stage tumors and estrogen-independent, late-stage, invasive tumors *in vivo*. In both model systems, the antitumor effect of Vitamin D analogs is associated with activation of apoptosis as well as inhibition of proliferation. In the metastatic model of breast cancer, Vitamin D analog treatment reduces the number of secondary tumors, suggesting that Vitamin D_3 analogs also inhibit the metastatic process. Current efforts in the laboratory are focused on identifying determinants of cellular sensitivity to Vitamin D and the direct targets of Vitamin D in breast tumors.

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It has been 15 years since scientists made the discovery of the VDR in cancer cells. Since then they have treated cancerous cells with Vitamin D to stop the tumors from growing. "In some cases this actually causes them to undergo a cell death process, called apoptosis," she said. Just what are the underlying mechanisms for Vitamin D's action in these cancerous cells?

On the molecular level, Vitamin D turns on genes that cause cancer cells to stop growing. "And so we've been using a lot of different approaches to study that," Welsh said. "We actually first characterized the biology of the cells—stopping the proliferation and then undergoing the cell death process. We are just seeing which pathway the cells take when they undergo apoptosis. We know other agents that produce cell death, and so we are comparing what we see in Vitamin D treatment to see if there are similar pathways." Are there changes in protein expression that might trigger this process?

Many pharmaceutical firms have invested millions of dollars in developing analogs of Vitamin D to treat various metabolic conditions related to bone function. "We are trying to develop a compound that will not have side effects on bone and yet will have an anticancer effect," she said.

1,25-dihydroxycholecalciferol activates VDRs in the central nervous system, skin and hair follicles, the immune system, and endocrine glands. It is the basis for the Vitamin D-based drug, Dovonex. Dovonex is applied topically to halt psoriasis, in which skin cells proliferate out of control.

The wealth of scientific data on the effects of Vitamin D still doesn't answer one very basic question: "What is VDR doing in the cells of mammary glands?" After pregnancy and after lactation, there is a regression of the mammary gland back to its normal state. "Essentially breast tissue has to be remolded back to a nonproliferating state," Welsh said.

The answer lies with apoptosis, or cell death. "This is the best example of natural apoptosis in the body," she said.

So, Welsh explained, if Vitamin D is important in the reproductive life cycle of the mammary gland, then would she see differences in gland function if Vitamin D action were somehow impaired?

Vitamin D-mediated processes in mammary gland.

The VDR is present in normal mammary epithelial cells as well as in breast cancer cells; however the role of Vitamin D signaling in mammary gland has not yet been defined. Because Welsh's studies have demonstrated that Vitamin D signaling can inhibit growth of breast cancer cells, she has hypothesized that Vitamin D may also regulate proliferation, survival, or functional differentiation of the epithelial cells in the normal mammary gland. If so, then VDR may act to suppress tumorigenesis. Using organ-culture approaches, Welsh's lab has found that 1,25D inhibits estrogen + progesterone-driven proliferation in mouse mammary glands. Further, using a VDR knock-out mouse model, they have shown that disruption of Vitamin D signaling alters development of the mammary gland during puberty, pregnancy, lactation, and weaning. These studies indicate that lack of functional VDR impacts on cellular turnover in the gland during normal development and suggest that Vitamin D signaling may reduce susceptibility of mammary cells to carcinogenesis. This concept has been tested by breeding VDR knock-out mice onto a transgenic mouse model of breast cancer (MMTV-Neu mice), and the results indicate that loss of VDR signaling reduces tumor latency and enhances tumor incidence. Further, Welsh's lab has demonstrated that lack of the VDR also enhances the development of skin tumors. These data from Welsh's lab have provided the first evidence that the VDR plays a role in regulation of cellular proliferation and differentiation *in vivo*, and the Vitamin D endocrine system impacts on susceptibility to cancer development.

She launched a study using a strain of bioengineered "knock-out" mice in which the VDR has been deleted. Ordinarily, the lack of the VDR makes for one sick animal. "But what we are able to do is to give these mice a very high-calcium diet that enables the bones to grow and the animal to develop normally," she said.

Welsh and her students have been looking at the mammary gland and how it develops in the absence of the VDR compared to mice having the receptor.

When they examine the knock-out mice during various stages of pregnancy and involution, they study the gland and look for changes in gene expression or changes in function and the composition of the milk.

Just as Welsh surmised, significant differences occurred in the mammary gland function of normal mice with the VDR and the knock-out mice.

It stands to reason that without Vitamin D regulating cellular growth, the mammary glands from knock-out mice grew at a faster than normal rate "and that is what we have found," Welsh said. Knock-out mice would go through the lactation cycle normally, but then at the end of the cycle the opposite would happen: without the VDR, apoptosis was delayed.

Welsh's studies indicate that Vitamin D plays an important role in cell death and cell proliferation in both cancerous and normal tissues. What the Notre Dame researchers don't know is what initial genes are turned on by Vitamin D. Are they different from other treatments that promote cell death or cell proliferation?

Vitamin D research has yet to resolve important questions. What investigators like Welsh need to understand is whether Vitamin D alters processing or distribution of proteins in the cell. Certainly, the VDR turns genes on. "More important is how proteins are processed and where they are in the cell and how they are regulated," Welsh said. Then it may become clearer how Vitamin D suppresses out-of-control cell proliferation in tumors.