Invited Comment

Apoptosis: background and possible role in secondary hyperparathyroidism

T. B. Drüeke, P. Zhang*, J. Gogusev

Unité 90 de l’INSERM and Division of Nephrology, Hôpital Necker, Paris, France

General features of apoptosis

In living organisms, programmed cell death or apoptosis is a critical physiological feature of regulated development and the subsequent homeostatic control of cell numbers in most, if not all tissues. Cells have the ability to self-destruct by activation of an intrinsic cell suicide programme when they are no longer needed or have become seriously damaged [1]. The basic machinery to carry out apoptosis appears to be present in essentially all mammalian cells at all times, but the activation of the suicide programme is tightly regulated by a cascade of signals and molecular events. The execution of this programme is often associated with characteristic morphological and biochemical features, including changes in the plasma membrane such as loss of membrane asymmetry and attachment, cell shrinkage, chromatin condensation, and chromosomal DNA fragmentation. The process probably starts by proteolytic activation of the cytosolic cysteine protease, caspase-3 which in turn activates DNA fragmentation factor (DFF) to induce nuclear DNA fragmentation [2]. The dying cells often fragment into membrane-bound apoptotic bodies that are rapidly phagocytosed and digested by macrophages or by neighbouring cells. Note that programmed cell death is a rapid process, the completion of which may require from 20 min to 24 h.

Apoptosis has to be distinguished from necrosis, which is an accidental, pathological form of cell death in response to injury. Necrosis is accompanied by cell swelling and lysis. This leads to the release of cytoplasmic material into the extracellular space, which often triggers an inflammatory response.

The initiation of apoptosis is carefully regulated. A number of signals are involved that may originate from both the intracellular and the extracellular milieu. These include lineage information, cellular damage inflicted by ionizing radiation or viral infection, extracellular survival factors, cell interactions, and hormones. In addition to the beneficial effects of programmed cell death, its inappropriate activation may cause or contribute to a variety of diseases, including AIDS, neurodegenerative diseases, ischaemic stroke, and cardiac failure [1,3].

The identification of a steadily increasing number of new molecules that influence cell survival in either a positive or a negative fashion, is a permanent challenge for the clinician who attempts to keep pace with the rapid developments in this field. A more precise insight into the apoptotic process and its physiological and pathological triggers will allow improvement in the understanding of disease. In addition, it may eventually open new avenues of diagnosis and treatment, such as the recently described bcl-2 antisense therapy in patients with non-Hodgkin lymphoma [4].

Bcl-2 family

One of the best-studied survival genes is bcl-2, the first member identified of the growing family of genes that participate in the control of apoptosis. The bcl-2 gene family contains both anti-apoptotic and pro-apoptotic members [5]. Table 1 presents the action of the Bcl-2 related proteins schematically. The Bcl-2 family members have the capacity to dimerize. Thus Bad and Bax can dimerize with Bcl-2 and Bcl-xL. Overexpression of Bad counters the survival promoting effect of Bcl-2 and Bcl-xL. The ratio of Bcl-2 to Bax appears to determine the fate of transfected cells, an excess of Bcl-2 resulting in survival but an excess of Bax resulting in death. However, the situation is complicated by the finding that pairs of anti-apoptotic members can also form heterodimers. Moreover, depending on the cell system, some family members can promote either cell death or cell survival. At least two different models of interaction have been proposed recently, in which Bcl-2 and Bcl-xL either actively repress cell death or else heterodimerize with Bax to prevent the formation of toxic Bax homodimers [6]. In the latter model, the additional presence of Bad would then disrupt Bcl-2-Bax and Bcl-xL-Bax heterodimers, liberating Bax once again to self-dimerize and promote death.
Agents involved in the regulation of cell survival

Growth factors and cytokines

Several growth factors participate in the regulation of cell survival. Thus IL-2, IL-4 and IL-7 enhance the survival of resting T-cells, GM-CSF and IL-3 that of myeloid cells, erythropoietin that of erythroid precursors, and IGF-1 that of fibroblasts, mostly in a Bcl-2-independent fashion. At least two major signal transduction mechanisms are involved, that is tyrosine kinases and the guanine nucleotide binding protein Ras, with Raf-1 being the best characterized downstream Ras effector. Disturbingly, Ras is capable of activating contradictory intracellular pathways that modulate cell viability, through the activation of either Raf-1 or phosphatidylinositol-3-kinase (PI(3)K) [7]. Other cytokines trigger apoptosis, including Fas ligand (FasL) and TNF. The latter acts by activating the cysteine protease, CPP32 (also named caspase-3) via a mitochondria-dependent pathway [8], probably through the release of cytochrome c. Interestingly, the mitochondrial release of cytochrome c has recently been shown to be a major target for the anti-apoptotic effects of Bcl-2 [9,10]. FasL, another member of the TNF cytokine family, is a potent inducer of either proliferation or apoptosis. When binding to its cell surface receptor Fas, it induces dimerization of the receptor and activates a cascade of intracellular reactions leading to cell proliferation or differentiation. On the other hand, FasL binding can also induce receptor trimerization, which then can transduce the death signal [11].

Calcium, vitamin D and retinoids

Abnormal calcium metabolism has long been known to be a common pathway for cell injury and cell death [12,13]. Several studies have shown that Ca$^{2+}$ may take part in almost every step in the course of induction of apoptosis. Direct evidence has been obtained that increases in cytoplasmic Ca$^{2+}$ can mediate apoptonuclease activation and cell death, whereas intracellular Ca$^{2+}$ buffering agents and extracellular Ca$^{2+}$ chelators inhibited both DNA fragmentation and cell death [14]. The depletion of intracellular Ca$^{2+}$ stores and also an enhanced influx of Ca$^{2+}$ across the plasma membrane can promote sustained increases in intracellular free Ca$^{2+}$ which can act as a signal for apoptosis, perhaps in part by activating key catabolic enzymes such as proteases, endonucleases, and transglutaminases. Thus evidence has been provided in favour of an involvement of Ca$^{2+}$-activated apoptosis in interleukin-3-dependent mouse haemopoietic cells [15], in mouse lymphocytes treated by glucocorticoids [16] and in rat liver [17]. In the latter work it was shown that incubations of isolated rat liver nuclei with ATP, NAD$^{+}$ and submicromolar Ca$^{2+}$ concentration resulted in extensive DNA hydrolysis. The Ca$^{2+}$-mediated DNA fragmentation is calmodulin dependent, since it can be blocked by the calmodulin antagonist, W7 in human CD4$^+$ cells expressing gp160 of HIV-1 [18].

Calcitriol is not only a cell-growth-inhibiting and differentiation-inducing agent, but also a promoter of programmed cell death, as shown by morphological and biochemical evidence in diverse tissues and cells [19–23]. A possible implication of vitamin D in apoptosis has been suggested by others to account at least partially for its growth-inhibitory action [24,25]. Thus the treatment of the human breast cancer cell line, MCF-7 with calcitriol reduced the level of bcl-2 expression [25]. The action of this active vitamin D$^3$ metabolite could be mediated by a depletion of intracellular calcium stores and the subsequent increase in cytoplasmic free Ca$^{2+}$ concentration, thereby inducing apoptosis [24]. Finally, 20-epi-vitamin D$^3$ analogues have been shown to inhibit proliferation and to induce apoptosis in human breast cancer cells through both up-regulation of p53 and down-regulation of Bcl-2 expression [19].

Retinoids, like active vitamin D derivatives, are also capable of inhibiting proliferation and of inducing differentiation, by binding to retinoic acid receptor (RAR), but apparently cannot induce apoptosis through this pathway. Induction of apoptosis may require ligand activation of retinoid X receptors (RXR) [26]. A more potent induction of terminal differentiation and programmed cell death, probably via RXR heterodimer formation, can be achieved by the combination of a 20-epi-vitamin D$^3$ analogue with 9-cis-retinoic acid [27].

Since Bel-2 is able to induce increases in both cytosolic and intranuclear Ca$^{2+}$ [28,29], and since it either directly or indirectly regulates transmembrane Ca$^{2+}$ flux at the level of endoplasmic reticulum, thereby controlling Ca$^{2+}$ signalling of apoptosis [30], one could put forward the hypothesis that calcitriol possibly modulates target cell apoptosis by modifying
intracellular free Ca\(^{2+}\) and thereby interfering with Bcl-2 action. However, cytoplasmic Ca\(^{2+}\) may also induce apoptosis in a Bcl-2-independent, but TGF-\(\beta\)-dependent fashion [31].

**Practical assessment of apoptosis: technical aspects and pitfalls**

Several methods have been applied to assess apoptosis either on tissue sections or in isolated cells grown in culture, including cell counting, flow cytometry, and various markers of apoptotic cells. However, methodological studies evaluating sources of error in the assessment of apoptotic cells have been rare until recently. With increasing knowledge of the complexity of the genetic pathways involved in regulating and executing programmed cell death it is now apparent that many of the changes in nuclear DNA structure, cytoskeleton, and antigen expression may be peculiar to certain cell types or specific apoptosis triggered pathways. Thus, a healthy degree of scepticism should be exercised when confronted with any novel assay [32].

**Morphological techniques**

The most ancient method used to assess apoptosis is based on morphological appearances. It consists of counting apoptotic cells, which can be easily recognized on haematoxylin–eosin stained tissue sections by applying strict morphological criteria. A systematic sampling protocol must be followed and cells should be counted at a relatively high magnification to obtain acceptable reproducibility [33]. There are, however, many objections to this, including doubts as to the specificity, the problems of how to count a fragmented cell, and the sensitivity of the technique [32].

**In situ techniques**

Assays based on nuclear DNA fragmentation have gained widespread use for identifying apoptotic cells. DNA fragmentation results from activated endonucleases that degrade the chromatin structure into fragments of 50–300 kilobases, and subsequently into smaller DNA fragments of about 200 basepairs in length. The *in situ* end-labelling (TUNEL) and *in situ* nick translation (ISNT) techniques, which have been developed during recent years, rely on the presence of DNA strand breaks characteristic of the nuclear implosion and fragmentation seen in apoptosis. For the latter, binding of labelled deoxyribonucleotide triphosphates (dUTP) to 3'-hydroxyl ends of double and single-stranded DNA is accomplished by terminal nucleotidyl transferase (TdT). Labelling of dUTP can be obtained with small ligands such as Br (Br-dUTP) or larger ligands such as fluorescein, biotin or digoxyginin. Several kits are available at present allowing to label DNA fragments and enabling detection of apoptosis by fluorescence or light-microscopy counting.

The accessibility of DNA breaks for enzymatic reactions is conditioned by the nuclear protein environment. This main drawback for TUNEL is worsened by cell and tissue fixation [34]. Therefore, for chemical pretreatment of cell lines, the most appropriate method, not only in terms of sensitivity but also in terms of specificity, appears to be microwave pretreatment, whatever the fixation technique used [35]. However, this method is not necessarily optimal for fixed tissue sections. Obviously the best pretreatment method must be elaborated for each type of tissue to be examined. Generally speaking, a number of critical criteria must be used to obtain a sensitive and specific evaluation of apoptosis by the TdT assay, including the following [35]: for comparative quantification, avoid using samples fixed according to various modalities and standardize pretreatment; accept labelling as specific only if it is strong compared with background; exclude isolated cells having mitotic or necrotic character; use automated methods such as flow cytometry or morphometric counts.

**Antigenic markers**

Protein markers of the apoptotic process have been developed which permit ready detection by immunohistochemistry. They include transglutaminase expression in liver cell apoptosis, anti-apoptotic specific protein (ASP) antibody in lymphoma and enhanced expression of CD11b/CD18 and CD11c/CD18 in apoptotic neutrophils. These markers probably are cell-lineage and apoptotic-trigger specific and may be useful only when properly validated in the specific cell lineage being studied [32]. A powerful new method is based on the observation that changes of the plasma membrane of the cell surface are one of the first steps of apoptosis, with phosphatidyl serine being translocated from the inner to the outer side of the plasma membrane. This process exposes the phospholipid to the extracellular milieu, thereby favouring the binding of external proteins such as annexin V [36]. Therefore the use of fluorescein-labelled annexin V has been proposed to identify early stages of programmed cell death [37].

**Hyperparathyroid disease states**

Obviously, both Ca\(^{2+}\) and calcitriol play a role at several steps of the apoptotic process. Diseases in which the handling of one or the other of these two agents is abnormal should therefore have some impact on programmed cell death. Consequently a theoretical basis for possible anomalies of apoptosis is given by the notion that in the various forms of primary (I°) and secondary (II°) hyperparathyroidism the concentration of circulating and cytoplasmic Ca\(^{2+}\) and/or calcitriol concentrations are generally disturbed. Moreover, their actions at the cell membrane, the cytoplasmic and the genomic nuclear level are frequently abnormal [38,39]. Finally, as to the II° hyperparathyroidism of chronic renal failure it is interesting to note that the uraemic state appears to induce a
higher rate of apoptosis in circulating monocytes [40], possibly via the well-known increase of cytosolic Ca\(^{2+}\) as shown in a variety of cells in renal failure [41]. What is the existing evidence for changes of apoptosis in hyperparathyroid diseases?

Before reviewing the contradictory results reported in the literature to date, it is useful to recall one of the basic features of normal parathyroid tissue growth. The parathyroid is an endocrine tissue whose proliferation rate is extremely slow; mitotic figures are seen extremely rarely [42]. This would indicate that under physiological steady-state conditions, at least in adult life, the rate of apoptosis should be comparably low. However, it should be measurable if sufficiently sensitive techniques are employed, because even a normal proliferation rate can successfully be determined with various detection methods [43–45].

Two research groups recently examined the rate of apoptosis in normal rats, as well as in rats that had been submitted to various stimuli of parathyroid tissue proliferation, such as low-calcium or high-phosphate diets. They were unable to find any evidence of programmed parathyroid cell death [44,45]. This was true not only for normal, but also for hyperplastic tissue. One explanation for this failure could be lack of sensitivity of the employed methods, especially when considering that apoptosis occurs very rapidly [45]. Both groups used the sensitive DNA nick end-labelling technique, after prior protein digestion of tissue sections, with only minor technical differences between the two procedures. Another possibility is that the methods used were inappropriate for parathyroid tissue. We have found recently that protein digestion is extremely harmful to cryopreserved human parathyroid tissue. The failure of identifying any degree of apoptosis could thus be due to a problem of tissue preparation.

Four other groups reported more or less elevated apoptotic numbers in normal parathyroids and/or in primary or secondary uraemic hyperparathyroidism. The techniques used were either TUNEL [46–48] or flow cytometry [49]. It must be pointed out that three of the four reports, including our own, are only preliminary findings which have been presented at the 1996 meeting of the American Society of Nephrology and which still require confirmation.

Wang et al. [47] found evidence of apoptosis in 85% of I° adenomas. Three of the cases examined harboured more than 5% apoptotic cells. Apoptosis was also found in 13 of 15 remnants of normal tissue outside the adenoma capsule, as well as in two normal parathyroid glands removed from patients who underwent neck surgery for parathyroid-unrelated disease. These findings are similar to the preliminary observations made by our group [48], with 1.3%, 5.3% and 9.5% apoptotic cells respectively in normal parathyroid tissue, I° parathyroid adenoma and II° hyperparathyroidism. No normal parathyroid glands examined, though to a variable extent. It was also expressed in normal parathyroid tissue [47] and
in one-third of parathyroid carcinoma specimens [50]. Sugenoya et al. [46] found a significantly higher number of Bcl-2-positive cells in II° than in I° hyperparathyroidism whereas the number of Bax-positive cells was lower. The determination of the level of Bcl-2 expression was not felt useful in the differential diagnosis between benign and malignant forms of hyperparathyroidism [50].

From a theoretical point of view, an increase in the rate of apoptosis in hyperparathyroid states would allow to counteract the elevated proliferation rates which have been reported for both I° parathyroid adenoma [50,51] and II° uraemic hyperparathyroidism [52]. An enhancement of programmed cell death could also theoretically contribute to the regression of II° parathyroid hyperplasia which occurs generally, although not constantly for all parathyroid glands, after the correction of end-stage renal failure by a successful kidney graft. Whether parathyroid hyperplasia can regress under high-dose calcitriol therapy, as claimed by some [53] but not observed by others [43], and if so whether this occurs by enhanced apoptosis, diminished proliferation, or both remains an unsolved question which needs to be addressed with adequate exploration techniques.

The adequacy of techniques used to examine apoptosis is precisely the issue, not only for the parathyroids. Given present technical difficulties to demonstrate apoptosis in slowly growing tissues, it remains to be seen whether the above discussed apoptotic numbers are real or whether they result from technical artifacts, such as recently discussed for another human tissue, namely the myocardium [3]. In this study, using dUTP labelling, confocal microscopy, DNA gel electrophoresis and Bcl2-Bax expression to evaluate programmed cell death, an estimated value of roughly 0.25% apoptotic cells was found in myocardial tissue from patients with heart failure, compared with 0.001% in normal control myocardium. These numbers were 20- to 140-fold lower than in a previous study where programmed cardiomyocyte death was also evaluated in end-stage heart failure [54].

Our group as well as others are working hard at present to obtain credible and reproducible numbers for the estimation of apoptosis in both normal and pathologic parathyroid tissues. For this purpose, several different technical approaches must be chosen and results compared with each other. Once the present technical difficulties have been overcome shall we be able to provide a reliable evaluation of the precise role apoptosis may play in the regulation of normal and abnormal parathyroid cell growth.

References

7. Mehta K, McQueen T, Neamati N, Collins S, Andreff M. Activation of retinoid receptors RARα and RXRα induces differentiation and apoptosis, respectively. Cell Growth Different 1996; 7: 179–186
28. Bally G, Miyashita T, Williamson JR, Reed JC. Apoptosis induced by withdrawal of interleukin-3 (IL-3) from an IL-3-dependent hematopoietic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced bcl-2 oncoprotein induction. *J Biol Chem* 1993; 268: 6511–6519


45. Wang Q, Palnitkar S, Parfitt AM. Parathyroid cell proliferation in the rat: effect of age and of phosphate administration increases both size and number of and recovery. *Endocrinology* 1996; 137: 4558–4562


