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Role of Intracellular pH in Proliferation, Transformation, and Apoptosis

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Both cellular proliferation and apoptosis (programmed cell death) have been claimed to be modulated, perhaps even triggered by, changes in intracellular pH. In this review, we summarize the evidence that gave rise to these hypotheses. To facilitate a critical appraisal of the existing data, we briefly review the main pathways involved in cytosolic pH homeostasis and their regulation by mitogens and by apoptosis-inducing agents. The information available at present suggests that cytosolic pH plays a permissive role in cellular growth and proliferation, but is neither a trigger nor an essential step in the mitogenic signal transduction cascade. Concerning apoptosis, it is clear that lowering the pH *in vitro* can activate DNase II. However, the evidence linking cytosolic acidification with DNA degradation *in vivo* is presently not convincing. We conclude that the cytosolic pH, an essential physiological parameter that is tightly controlled by multiple, complementary, or redundant systems, is unlikely to play a role in signalling either cell growth or death.

KEY WORDS: Na^+/H^+ exchange; proton pump; V-ATPase; anion exchange; cancer.

TUMOR pH AND THERAPY

Effective prevention and treatment of human cancer requires full understanding of the parameters that determine cell growth, proliferation, and death. One of the factors thought to control the rate of proliferation and the onset of programmed cell death is the cytosolic pH. In the following we will discuss the significance of the intracellular pH (pH_i) and of the transport processes responsible for its homeostasis in relation to cellular proliferation, transformation, and apoptosis.

Comparatively crude, macroscopic measurements of pH have shown that the medium surrounding solid tumors is generally in the acidic range, though individual results can vary widely (see Griffiths, 1991 for review). The pH recorded is not only dependent on the type of tumor, but has also been found to vary spatially within the heterogeneous microenvironment of malignant tumors (Rockwell and Hughes, 1990). Extracellular accumulation of lactate is thought to be a major cause of tumor acidity. Regional hypoxia caused by variable blood flow and metabolism cause lactic acid generation in areas of tumors where anaerobic metabolism predominates. Furthermore, chemo- and radio-therapy influence the microenvironment of tumors and, conversely, regions with hypoxia and acid extracellular pH within a tumor influence the effectiveness of therapeutic regimes (Durand, 1991). Based on the unique microenvironment of solid tumors, treatment modalities designed to manipulate pH have been repeatedly suggested and applied clinically. Furthermore, rare instances of spontaneous regression of tumors have been speculatively ascribed to alterations of pH (Harguindey and Cragoe, 1992).

By extension of the findings made measuring macroscopic (largely extracellular) pH, the assumption was made that the cytosolic pH of tumor cells was also acidic. However, the advent of novel techniques for noninvasive measurement of intracellular pH has disproved this notion: the cytosolic pH of tumor cells

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is generally *not* acidic (Stubbs *et al.,* 1995). Importantly, in the face of the well-documented acidic extracellular pH of tumors, tumor cells must be able to extrude protons more efficiently than their normal counterparts in order to maintain the cytosol near neutrality. In fact, the cytosolic pH of transformed cells has been often reported to be more alkaline than that of normal cells.

pH-HOMEOSTASIS DURING CELL GROWTH AND TRANSFORMATION

Cell transformation causing dysregulation of cell proliferation and/or cell death is a prerequisite for the development of a cancerous lesion. A causative link between cellular pH homeostasis and tumor development has been repeatedly suggested, and an elevated cytosolic pH has been demonstrated to parallel both cell transformation and proliferation (Doppler *et al.,* 1987; Hagag *et al.,* 1987; see also below). Therefore, it is important to understand the mechanisms whereby transformed or proliferating cells upregulate their capacity to extrude proton equivalents. Clearly, such knowledge could provide new targets for intervention in conditions where selective impairment of the growth of a subpopulation of cells is desirable, as in the case of cancer.

Cells have multiple mechanisms for pH regulation that are often regulated in concert (see Fig. 1). Such a redundancy of regulatory mechanisms most likely reflects the crucial importance of pH maintenance for overall cell function and survival. The following sections discuss the main pH homeostatic mechanisms that have been invoked in the control of cell proliferation and survival, with particular emphasis on the more recent literature. For an extensive review of earlier work pertaining to pH and cell growth the reader is referred to Grinstein *et al.* (1989).

PROTON PUMPS

All cells express vacuolar (V)-type proton pumps in endosomal, lysosomal, and Golgi compartments. In addition, a restricted number of cell types (e.g., urinary epithelial cells, osteoclasts, macrophages) express in their plasmalemma V-type proton pumps that contribute to cytosolic pH-regulation, transepithelial solute transport, and acidification of sealed-off extracellular spaces (Forgac, 1989). Proton pumping through the V-ATPase can be readily demonstrated by virtue of its

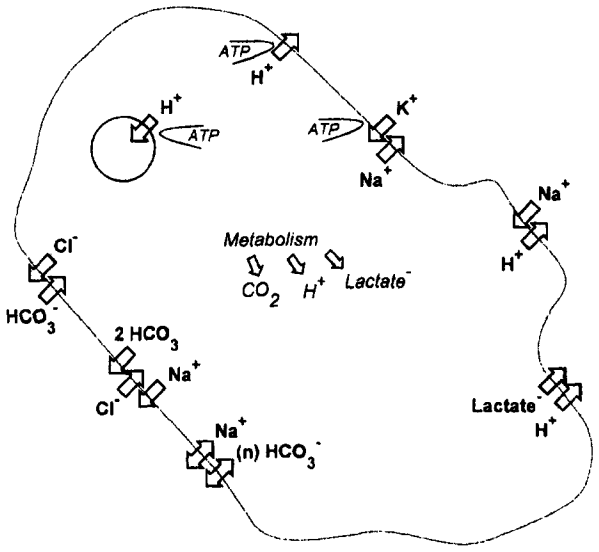

Fig. 1. Diagrammatic summary of the main acid/base transporters of mammalian cells. Vacuolar H^+-ATPases, Na^+/H^+ exchangers, lactate$^-$-H^+ symporters, and cation-dependent and independent anion exchangers are illustrated. The sources of metabolic acid, CO_2, lactate, and the Na^+/K^+ ATPase are also included. An endomembrane organelle with vacuolar H^+-ATPases is also shown.

specific and effective inhibition in the presence of the macrolide antibiotics bafilomycin and concanamycin. The regulation of this multi-subunit proton transporter is poorly understood, but three separate lines of evidence suggest that dysregulation of proton pumping across the plasma membrane might be linked to a cancerous phenotype. First, transfection with a yeast plasmalemmal H^+-ATPase was shown to induce transformation of mammalian cells, possibly by elevating the cytosolic pH (Perona and Serrano, 1988; Gunn *et al.,* 1994). Second, the relatively high cytosolic pH of some tumor cells has been correlated with and attributed to bafilomycin-sensitive proton transport across the surface membrane (Martinez-Zaguilan *et al.,* 1993). Third, a transforming viral oncoprotein was shown to bind to a subunit of the V-ATPase both *in vivo* and *in vitro* (Goldstein *et al.,* 1991). However, the following caveats should be considered: (i) The heterologous expression of a yeast proton pump in mammalian cells may have effects other than those directly related to alterations in the cytosolic pH. (ii) Bafilomycin-sensitive regulation of pH was demonstrated only in a minority of tumor cell lines tested. (iii) The functional consequences of oncoprotein interaction with the V-ATPase, in particular its effect on pH, have not been established. Thus, one must conclude that the evidence linking V-ATPase activity to

cell transformation, however tantalizing, is presently incomplete.

THE Na^+/H^+ EXCHANGER

In the 1980's, it was widely believed that an elevation of cytosolic pH was causally related to cellular proliferation and might be responsible for transformation. The prevailing view was that such an alkalinization was caused by activation of the Na^+/H^+ exchanger (NHE). Several advances have made this hypothesis amenable to direct analysis. First, five isoforms of the exchanger have been identified and sequenced and, as a result, our knowledge of the molecular details of NHE and its regulation has rapidly expanded (the reader is referred to a recent review on the topic; Wakabayashi *et al.*, 1997). The NHE isoforms differ in tissue distribution and have multiple cellular functions including pH homeostasis, cell volume regulation, and transcellular solute transport. Additionally, the ubiquitous isoform NHE1 has been implicated in carcinogenesis since its increased activity has been associated with proliferation, differentiation, and neoplastic transformation.

Activation of receptor tyrosine kinases and G protein-coupled receptors can stimulate the antiporter by shifting its sensitivity to $[H^+]$, and phosphorylation has been suggested to play a role in its regulation. Constitutive activation of the same signaling pathways is associated with oncogenesis, suggesting a link between these events. Indeed, treatment of the cells with mitogens activates and induces phosphorylation of NHE1. Furthermore, the exchanger is a calmodulin-binding protein, and calcium/calmodulin appears to regulate the activation of NHE1. Therefore mitogenic agents may also stimulate NHE1 by a phosphorylation-independent mechanism (McSwine *et al.*, 1996). In addition, the existence of accessory factors that can interact and modulate the activity of NHE1 has been postulated (Goss *et al.*, 1994; Wakabayashi *et al.*, 1997).

Heterotrimeric and/or small GTP-binding proteins are also thought to regulate the function of NHE1. Recently, activation of NHE1 by GTP-binding proteins was demonstrated to occur through multiple distinct signaling pathways (Hooley *et al.*, 1996): Ras was shown to stimulate the exchanger through a MEK-mediated pathway, whereas activation by $G\alpha_{13}$ occurred through MEKK and simultaneously through a separate Rho-mediated pathway.

Conversely, it was shown recently that NHE activity is necessary, but not sufficient, for Rho-induced stress-fiber formation (Vexler *et al.*, 1996). GTPases of the Rho-family are known as regulators of actin cytoskeletal architecture (Nobes and Hall, 1995), and of cell anchorage to the substratum. Because it is well established that the anchorage dependence for growth observed in normal cells is subverted in transformed cells, it is attractive to speculate that NHE1 may play a role in this conversion. This possibility is at present highly speculative and awaits confirmation of the role of NHE in anchorage and understanding of the basis of the loss in anchorage dependence in malignant cells.

Early studies demonstrated that activation of the NHE was required for cell growth and proliferation. However, these studies were often performed under conditions precluding the operation of other pH regulatory mechanisms, e.g., in the nominal absence of bicarbonate. It was therefore conceivable that the inability of the cells to grow reflected simply an abnormal intracellular pH, which is required for the proper function of most enzymatic processes. In support of this concept, antiporter-deficient mutants (expressing no detectable amounts of NHE protein) were found to grow at normal rates, provided bicarbonate is included to support normal pH regulation by alternative pathways (see below). These mutants were unable to proliferate at acidic extracellular pH, implying that unphysiological intracellular pH precludes growth. Transfection of antiporter-deficient cells with the epithelial isoforms (NHE2, NHE3) of the exchanger supports proliferation at acidic pH (Kapus *et al.*, 1994), indicating that the ubiquitous "housekeeping" NHE1 isoform is not essential for cytosolic pH regulation and cell growth. It is noteworthy that the responsiveness of these isoforms to mitogens differs from that of NHE1. Since all the different isoforms are activated by cytosolic acidification, one must conclude that the basal activity of the different isoforms, and not their response to mitogens, is essential for cell growth. That an increase in NHE1 activity is not essential was also suggested by the ability of active $G\alpha_{12}$ to inhibit NHE1, while nevertheless stimulating cell proliferation and inducing neoplastic transformation (Lin *et al.*, 1996).

In conclusion, we believe that the role of NHE in proliferation and carcinogenesis is permissive, rather than causative. In other words, the exchanger is not intimately involved in the genesis or transmission of signals that lead to cell growth or transformation. Instead, maintenance of a neutral or slightly alkaline

cytosolic pH is required for these events and the exchanger is central to pH homeostasis, particularly when other regulatory pathways have been disabled by removal of bicarbonate.

BICARBONATE AND LACTATE TRANSPORT MECHANISMS

Metabolic by-products, including CO_2/H_2CO_3, are retained in the vicinity of poorly perfused solid tumors. Lactate is extruded from cells together with protons in order to maintain electroneutrality, thereby acidifying the extracellular space. However, the acid pH of tumor tissue can only be ascribed in part to lactate extrusion (Newell *et al.*, 1993), and extracellular carbonic acid accumulation might contribute as well. Because of the abundance of HCO_3^- (derived from carbonic acid) in the vicinity of solid tumors, one might expect that regulation of pH in cells from these tumors may rely extensively on the transport of this anion.

To defend their cytosolic pH, most cells express both acid-extruding and acid-loading HCO_3^- transporters. These molecules catalyze the transport of HCO_3^-/CO_3^{2-} coupled to the transport of Na^+ and/or Cl^-. These mechanisms are exquisitely sensitive to the cytosolic pH, and this pH-dependence of their activity can be shifted upon activation of the cells by agonists, as described above for the NHE. In mesangial cells, growth factors have been shown to stimulate both alkalinizing and acidifying HCO_3^--dependent mechanisms (Ganz *et al.*, 1989). An enhanced ability of the cells to regulate pH can be envisaged to facilitate the maintenance of conditions that would be permissive for the proliferative response. In other words, by stimulating HCO_3^- transport, mitogens may enhance the ability of cells to regulate cytosolic pH in the face of an enhanced metabolic burden. Unfortunately, the effect of mitogens or of transformation on the HCO_3^--dependent mechanisms has not been extensively studied. Nevertheless, in Ras-transformed fibroblasts an alkalinization was noted, which was shown to be caused by an altered pH dependence of both NHE and Na^+-dependent Cl^-/HCO_3^--exchange (Kaplan and Boron, 1994). Additional experimentation is required to better evaluate the role of anion transporters in pH regulation during mitogenesis and transformation.

INTRACELLULAR pH AND APOPTOSIS

Uncontrolled proliferation of cells can be caused not only by excess growth, but also by reduced rates of cell death. Apoptosis, or programmed cell death, is biochemically characterized by DNA fragmentation. Early studies indicated that DNase I, a calcium-dependent endonuclease, was responsible for the characteristic pattern of DNA degradation (DNA ladder) observed in apoptotic cells (Wyllie *et al.*, 1992; Ellis *et al.*, 1986). However, not all cells contain DNase I, and not all apoptosis models exhibit increases in free cytosolic calcium. Likewise, intracellular calcium levels in apoptotic cells often do not reach levels necessary for calcium to mediate DNase I activity. Therefore, another nuclease must also play a role in mediating DNA fragmentation in apoptotic cells.

There have been several reports that associate intracellular pH and apoptosis (Weinberg *et al.*, 1994; Barbiero *et al.*, 1995; Gottlieb *et al.*, 1996). Recent studies have linked a pH-dependent endonuclease, DNase II, to apoptosis (Barry and Eastman, 1992; Barry *et al.*, 1993; Perez-Sala *et al.*, 1995). First, a correlation between acidification and DNase II endonuclease activity was observed when apoptosis was induced by either ionomycin or lovastatin in HL-60 cells (Barry and Eastman, 1992; Perez-Sala *et al.*, 1995) or by removal of interleukin-2 (IL-2) from the medium bathing CTLL-2 cells, a T cell line dependent on IL-2 for survival (Rebollo *et al.*, 1995). For example, intracellular pH in a population of apoptotic HL-60 cells decreased by 0.2–0.3 (Barry and Eastman, 1992). Likewise, pH_i in CTLL-2 cells decrease from pH 7.5 to 7.0, upon lovastatin-induced apoptosis (Rebollo *et al.*, 1995). Second, overexpression of Bcl-2, a protein that protects against apoptosis, attenuated acidification, and subsequently prevented apoptosis in Chinese hamster ovary cells and Jurkat T lymphoblasts (Reynolds *et al.*, 1996; Meisenholder *et al.*, 1996). Therefore, a link between acidification and DNA fragmentation seems to exist, though the observed decreases in pH_i were relatively small.

This small decrease in pH_i reported casts doubt on the theory that DNase II is involved in apoptosis, since the optimal pH for DNase II activity (pH = 5.0) is considerably more acidic than the normal cytosolic pH (Eastman and Barry, 1992). At first glance, it would appear that the observed decreases in pH_i are not sufficiently large to account for the activation of DNase II. However, the pH_i, values reported in the preceding studies were averages of populations, possibly re-

flecting cellular heterogeneity. To circumvent this possibility, cells were separated by flow cytometry according to their pH_i. In two studies, an acidic subpopulation of cells had an average pH_i of 6.4–6.6 (Barry *et al.*, 1993; Perez-Sala *et al.*, 1995). More importantly, only cells in this acidic group displayed DNA fragmentation. While the average pH of these cells is at the upper end of the pH range required for DNase II activity, the fact that only acidic cells showed signs of DNA fragmentation establishes a link between acidification and apoptosis.

Growth factors, tumor promoters, and ionomycin often promote proliferation and protect cells against apoptosis. Granulocyte macrophage colony-stimulating factor (GM-CSF), IL-3, IL-2, and Steel factor protect against apoptosis in several cell types (Rajotte *et al.*, 1992; Caceres-Cortes *et al.*, 1994; Li and Eastman, 1995; Rebollo *et al.*, 1995). Phorbol esters and ionomycin suppress apoptosis in MOE-7 cells upon removal of growth factors (Rajotte *et al.*, 1992) and protect HL-60 cells from lovastatin-induced apoptosis (Perez-Sala *et al.*, 1995). The growth factors and tumor promoters that protect against apoptosis also cause intracellular alkalinization in several cell types. GM-CSF or IL-3 induce alkalinization in monocytes, HL-60 cells, endothelial cells, and MOE-7 cells (Vallance *et al.*, 1990; Schwartz *et al.*, 1991; Bussolino *et al.*, 1989; Rajotte *et al.*, 1992). In addition, phorbol esters induce alkalinization in U937 cells, neuroblastoma cells, and MOE-7 cells (Ng and Davies, 1991; L'Allemain *et al.*, 1994; Rajotte *et al.*, 1992). Growth factors often cause this alkalinization by activating the NHE (see above). Rajotte *et al.*, (1992) observed that growth factor- and phorbol ester-induced alkalinization in MOE-7 cells was inhibited by compound H7 and staurosporine, inhibitors of protein kinase C. Further, a functioning NHE had to be present for the phorbol esters to induce this alkalinization. Since growth factors and phorbol esters both protected against apoptosis in MOE-7 cells, it was suggested that the NHE may play a role in the protection against apoptosis.

To evaluate this possibility, MOE-7 and HL-60 cells were incubated with 5-(N-ethyl-N-isopropyl) amiloride (EIPA) or 5-(N,N-hexamethylene) amiloride (HMA), potent and reasonably specific inhibitors of the NHE (Rajotte *et al.*, 1992; Perez-Sala *et al.*, 1995). As little as 0.1 μM EIPA was sufficient to induce DNA fragmentation in MOE-7 cells, even in the presence of growth factor (Rajotte *et al.*, 1992). Similar results were obtained with 10 μM HMA. In the presence of 20–40 μM EIPA, DNA degradation occurred in HL-60 cells (Perez-Sala *et al.*, 1995). In addition, EIPA could preclude phorbol ester, GM-CSF, and Steel factor-induced protection against apoptosis, suggesting the NHE is required for this process (Rajotte *et al.*, 1992; Perez-Sala *et al.*, 1995; Caceres-Cortes *et al.*, 1994).

In addition, while GM-CSF and Steel factor both prevent apoptosis, they have different tyrosine kinase inhibitor sensitivity, yet have identical profiles of inhibition by EIPA (Caceres-Cortes *et al.*, 1994). This suggest that Steel factor, acting via the tyrosine kinase receptor c-Kit (Lev *et al.*, 1992), and GM-CSF, acting via Jak-2 (Stahl and Yancopoulos, 1993), have a point of convergence at the level of the NHE. If growth factors are removed, the exchanger is no longer activated, and an acidification occurs. Taken together, these results suggest the following model for protection against apoptosis. Growth factors, via a kinase C-dependent pathway, activate the NHE. The resulting alkalinization protects against apoptosis, presumably by maintaining pH_i at levels that prevent activation of DNase II (see Fig. 2).

While the above evidence supports the hypothesis that inhibition of the NHE is indeed involved in apoptosis, several lines of evidence suggest otherwise. First, and possibly most important, is the fact that acidification itself may not be required for apoptosis. HL-60 cells incubated in the absence of IL-2, but at an extracellular pH of 7.9, underwent only a modest acidification to 7.2, a pH_i that is normal or supranormal, yet still developed DNA fragmentation (Li and Eastman, 1995). Similarly, in CTLL-2 cells that were incubated at a high extracellular pH, staurosporine-induced apoptosis still occurred, despite the fact that intracellular pH did not fall below 7.2 (Reynolds *et al.*, 1996). Furthermore, thapsigargin-induced apoptosis in several androgen-independent prostatic cancer cell lines did not involve intracellular acidification (Furuya *et al.*, 1994). Therefore, if acidification is involved in apoptosis, it may not always be a necessary event.

The second line of evidence that contradicts the above hypothesis is the fact that several studies suggest that the NHE plays an *active* role in apoptosis, whereby intracellular *alkalinization* results in DNA degradation. Zhu and Loh (1995) showed that 5-(N,N-dimethyl) amiloride (DMA), another inhibitor of the NHE, induced acidification in HL-60 cells, yet no apoptosis was observed. They further demonstrated that *alkalinization,* induced by exposure to the sodium-proton ionophore monensin, resulted in apoptosis in HL-60 cells. In addition, DMA inhibited both Br-A23187-

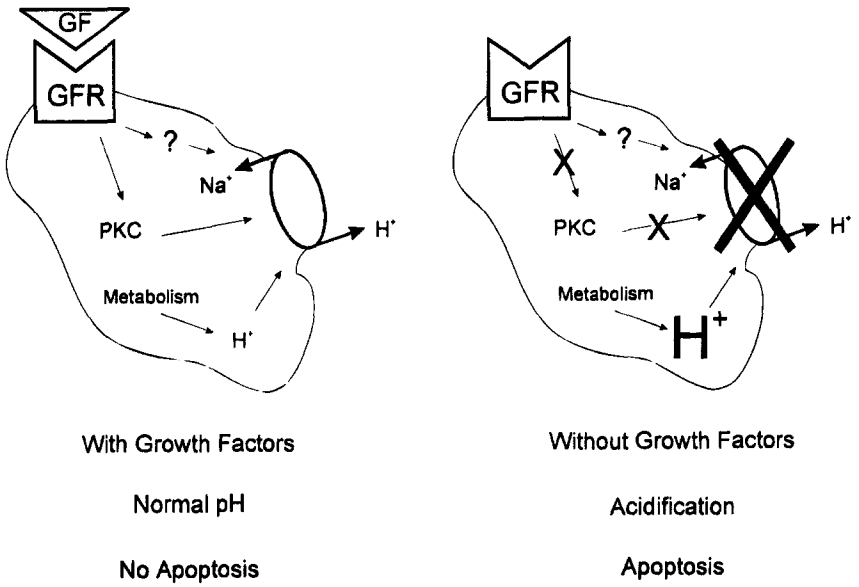

Fig. 2. Schematic representation of the model explaining the mechanism of prevention of apoptosis by growth factors (GF). Left panel: GF binds to its receptor (GFR), leading to activation of protein kinase C (PKC), which in turn maintains normal NHE activity, preventing the cells from accumulating metabolic acid and therefore undergoing apoptosis. Right panel: omission of growth factors depresses PKC activity, which in turn reduces NHE activity. The resulting accumulation of metabolic acid produces a drop in pH_i which activates DNase II and promotes apoptosis.

and thapsigargin-induced alkalinization and apoptosis in HL-60 cells (Zhu and Loh, 1995), suggesting both these agents promote apoptosis via a NHE-dependent pathway. These results contradict the previously discussed report in HL-60 cells that suggested the *inhibition* of NHE resulted in apoptosis (Perez-Sala *et al.*, 1995).

Further, Tsao and Lei (1996) have recently shown that in thymocytes, which spontaneously undergo apoptosis, the NHE, Na^+-HCO_3^- cotransporter and Cl^-/HCO_3^- exchanger, all of which normally have an alkalinizing effect on pH_i, are responsible for the observed apoptosis. A correlation between the degree of alkalinization and apoptosis existed in this study: a higher pH_i was associated with a higher percentage of apoptotic cells. In fact, significant increases in apoptosis were observed only when the pH_i was greater than 7.2. Recently, Wyllie *et al.* (1992) reported that an endonuclease with an optimum pH of 7.5 was involved in thymocyte apoptosis, as opposed to DNase II, which has an optimal pH of 5. Therefore, at least in thymocytes, alkalinization and not acidification, may be responsible for apoptosis.

Some of the discrepancies between the above investigations may be due to the wide variety of cell types used. Different cell types may have distinctive pathways that lead to a common convergence point, which eventually results in apoptosis. Conversely, different cell types may utilize different endonucleases in the process of DNA fragmentation.

In conclusion, several lines of evidence suggest a link between acidification and apoptosis. These findings led to the formulation of a model for protection against apoptosis, involving growth factor activation of the NHE via a protein kinase C-dependent pathway. The activation of the exchanger would protect the cells from apoptosis by maintaining pH_i above the levels that activate DNase II. Conversely, removal of growth factors would result in a decrease in NHE activity, and thus an acidification. Therefore, it has been suggested that growth factor protection against apoptosis is accomplished by maintaining a steady-state level of NHE activity. However, acidification itself appears not to be required for apoptosis in every instance. Moreover, other lines of evidence suggest that alkalosis, rather than acidification, may cause apoptosis in some cell types.

The available evidence is seemingly insufficient to conclusively relate pH_i to apoptosis. Additional experiments will be required to ascertain whether acid-

ification (or alkalinization) is sufficient or even necessary to effect apoptosis.

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