Apoptosis induced by Na⁺/H⁺ antiport inhibition activates the LEI/L-DNase II pathway

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Abstract

L-DNase II is derived from its precursor leucocyte elastase inhibitor (LEI) by post-translational modification. *In vitro*, the conversion of LEI into L-DNase II can be induced by incubation of LEI at an acidic pH. In this study, we proposed to analyze the effects of intracellular acidification on this transformation. Amiloride derivatives, like hexamethylene amiloride (HMA), are known to provoke a decrease of cytosolic pH by inhibiting the Na⁺/H⁺ antiport. In BHK cells, treatment with HMA-induced apoptosis accompanied by an increase in L-DNase II immunoreactivity and L-DNase II enzymatic activity. Overexpression of L-DNase II precursor led to a significant increase of apoptosis in these cells supporting the involvement of L-DNase II in HMA induced apoptosis. As previously shown in other cells, etoposide-induced apoptosis did not activate L-DNase. On the contrary, LEI overexpression significantly increased cell survival in etoposide-induced apoptosis. Together these results suggest differential roles of LEI and L-DNase II in response to different types of apoptotic inducers.

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Abbreviations: LEI, leucocyte elastase inhibitor; L-DNase II, LEI-derived DNase II; HMA, hexamethylene amiloride; DAPI, 4,6-diamidino-2-phenylindole; MTT, tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5 phenyltetrazolium bromide; pH_i, intracellular pH; TMACl, tetramethylammonium chloride; SNARF-1, carboxyl-seminaphthorhodafluor-1 acetoxymethylester.

Introduction

Apoptosis is a form of cell death implicated in many processes such as embryogenesis, normal tissue turnover and tumor regression. It is characterized by several morphological changes including membrane blebbing, cell shrinkage, phosphatidylserine externalization and chromatin condensation.¹ The ultimate hallmark is the digestion of genomic DNA into oligonucleosomal fragments. Many studies have been performed to identify the endonucleases responsible for this process. A number of cation-dependent endonucleases acting at neutral pH have been characterized such as Nuc 18/cyclophilin A,² DNase I,³ DNase γ ,⁴ and the 97 kDa DNase.⁵ The best characterized DNase in apoptosis is CAD, a DNase activated by caspases.⁶ DNase II, cation-independent endonucleases that are active at low pH, were also incriminated. It has been shown that the decrease of intracellular pH can activate DNase II.⁷ This activity has been isolated from diverse tissues and cell lines during apoptosis.⁸

Our group studies the leucocyte elastase inhibitor (LEI)/L-DNase II pathway that results in the activation of L-DNase II (LEI-derived DNase II). The activation of this DNase II (acid, cation-independent DNase) was first discovered in the lens during lens cell differentiation,⁹ which is an apoptosis-related cellular differentiation.¹⁰ The activation of this enzyme has also been seen in other physiological models such as neural apoptosis during retina development¹¹ or in cell culture.^{12,13} We have also shown that caspases do not participate in L-DNase II activation.¹⁴ Hence, the LEI/L-DNase II pathway is classified among the caspase-independent pathways.¹⁵ L-DNase II derives from its precursor LEI after a post-translational modification.¹⁶ The loss of LEI antiprotease activity and the appearance of L-DNase II endonuclease activity are accompanied by a decrease in its apparent molecular weight (LEI: 42 kDa; L-DNase II: 27 kDa).

LEI belongs to the ovalbumine structure subgroup of the serpins (serine protease inhibitor). Most of these serpins can inhibit target proteases and present diversified functions.¹⁷ Some regulate lysosomal proteinases (squamous cell carcinoma antigen), monocyte/granulocyte proteinases (proteinase inhibitor-6: monocyte neutrophil elastase inhibitor), fibrinolysis (plasminogen activator inhibitor 2) and bone marrow differentiation (bomapine). Others are tumor suppressors (maspin) or are implicated in angiogenesis.¹⁸ Several serpins can inhibit apoptosis. The viral serpin CrmA inhibits Fas or TNF α -induced apoptosis.¹⁹ Moreover, overexpression of PAI-2 or PI-9 protects cells from TNF α ²⁰ or granzyme B-induced apoptosis,²¹ respectively. This suggests that LEI, as a member of the serpin superfamily, could also have an antiapoptotic activity.

We have previously shown that under certain apoptotic conditions LEI is transformed into L-DNase II. Several authors have demonstrated that the overexpression of a DNase in a cell may induce apoptosis.^{22–25} In particular, DNase II

Figure 1 NHE antiport expression in BHK cells. (a) Total mRNA from BHK cells and from rat kidney were retrotranscribed from a poly(dT) primer and amplified by PCR using rat NHE1-4-specific primers. Signals are positive for NHE1, 2 and probably for NHE3. MW indicates the molecular weight markers (pb). (b) The NHE sensitivity to HMA was evaluated by measuring the effect of HMA on the capacity of NHE to re-establish intracellular pH after acidification induced by an NH_4Cl pulse. In the absence of HMA, the recovery rate was 4.7×10^{-4} upH/s. In the presence of $40 \mu\text{M}$ HMA this recovery rate was decreased to 2.0×10^{-4} upH/s

overexpression leads to apoptosis detected as chromatin condensation.²⁶ It may then be inferred that induction of DNase activity in a cell could induce apoptosis.

The dual functionality of LEI/L-DNase II led us to the hypothesis that LEI may act as a molecular switch between living cells and apoptotic cells.²⁷ Its dual functions may serve to prevent the proteolytic cascade of apoptosis in living cells while, after the transition to L-DNase II, releasing this proteolytic inhibition and inducing nuclear degradation in apoptotic cells.

A decrease in intracellular pH is a common effect of many apoptotic stimuli such as staurosporine,²⁸ somatostatin,^{29,30} cytokine deprivation^{20,31} and Fas-induced apoptosis.³² It has been suggested that intracellular acidification activates DNase II by providing optimal conditions for this enzyme.^{33–35} This could also be the case for L-DNase II. Since the transformation of LEI into L-DNase II can be catalyzed, *in vitro*, by acidic pH or elastase,¹⁶ we also hypothesized that acidic intracellular pH could cause the shift of LEI into L-DNase II during apoptosis. In this study, we induced intracellular acidification with hexamethylene amiloride (HMA), an Na^+/H^+ exchanger inhibitor, to explore this hypothesis.

Results

To better understand the function of the bifunctional molecule LEI/L-DNase II in apoptotic and living cells, it is convenient to modify the expression of this protein in order to evaluate the resulting cellular effects. We decided then to study the effect of LEI overexpression. From the ubiquitous expression pattern of LEI,³⁶ many cell types could potentially activate the LEI/L-DNase II pathway. Different cell lines were transfected with the expression vector pREP10 carrying the porcine LEI cDNA. Among these different cell lines, BHK cells showed the highest level of expression and the longest survival times (not shown) and were therefore chosen for this study.

Since LEI is transformed, in acidic conditions, *in vitro*¹⁶ and in some physiological models,³⁷ we hypothesized that a decrease in intracellular pH (pHi) may contribute to this

transformation. Among the different agents able to modify intracellular pH, the amiloride derivatives are very efficient in acidifying the cell by inhibiting the Na^+/H^+ exchanger (NHE gene family).³⁸ However, since little is known about this proton antiport in BHK cells, it was necessary to confirm NHE expression in these cells.

Na^+/H^+ exchanger expression in BHK cells

The NHE family contains seven members and the isoforms 1–4 are largely expressed in different tissues.³⁸ To identify the NHE isoforms expressed in BHK cells, total RNA was retrotranscribed and amplified by PCR. Sequence data for hamster NHE are limited since only the hamster NHE1 sequence has been published.³⁹ Since NHE1–4 were identified in rat kidney,⁴⁰ we used specific probes of rat NHE for PCR. The results shown in Figure 1 indicate that NHE1 is a major isoform expressed in BHK cells, a result that is in agreement with its ubiquitous expression.⁴¹ To further evaluate if these transporters are sensitive to HMA, we induced cellular acidification by an NH_4Cl pulse,⁴² and evaluated the rate of pH recovery in the presence and absence of HMA. As seen in Figure 1b, in the presence of HMA the rate of recovery was inhibited by HMA, indicating that this amiloride derivative is able to inhibit the activity of NHE in these cells. Moreover, if the intracellular pH in control cells was near neutrality (7.07 pH units) in the presence of HMA the intracellular pH decreased to 6.67, as measured by flow cytometry using the pH probe SNARF-1.

Induction of apoptosis in BHK cells by HMA

The intracellular acidification induced by blockage of the NHE exchanger by amiloride or its derivatives has been shown to induce apoptosis in many cell lines.^{43,44} To confirm such induction in BHK cells, we exposed these cells to $40 \mu\text{M}$ HMA for different periods of time⁴⁵ and estimated, 24 h after the start of treatment, the cell survival ratio by using a metabolic index provided by the MTT test. In these conditions, the survival rate significantly decreased with increases in exposure time and reached a mean of $6.8 \pm 2\%$ at 24 h of HMA treatment (Figure 2a).

Figure 2 HMA induces apoptosis in BHK cells. **(a)** BHK cells were incubated in DMEM medium containing 40 μ M HMA for 1, 2, 3, 4 or 24 h. After this period, the medium was replaced by conditioned medium obtained from seeded wells not treated with HMA. The percentage of surviving cells was calculated with the MTT method 24 h after the start of the treatment. Results represent the mean of three different experiments. $P < 0.001$ for all exposure times when comparing OD for treated and untreated cells. **(b)** Nomarsky photographs of BHK cells showing cell condensation and blebs in HMA-treated cells. Ctl: control cells; HMA: cells treated during 24 h. **(c)** After a 24-h treatment, BHK cells showed a ladder of DNA degradation (lane HMA), which is not seen in control cells (lane Ctl), MW indicates the molecular weight markers (pb). **(d)** Western blot of BHK cells incubated with HMA for different periods (0–24 h) developed with an anticaspase 3

Since the MTT test indicates cell survival and not apoptotic cell death, we investigated the type of HMA-induced cell death. In agreement with the cell survival rate presented in Figure 2a, most treated cells displayed membrane blebbing and apoptotic body formation (Figure 2b) after 24 h. In addition, DNA degradation pattern on agarose gel showed an internucleosomal ladder (Figure 2c). These results are in agreement with an apoptotic cell death induced by HMA. However, the induction of apoptosis by HMA did not induce caspase 3 activation (Figure 2d). Western blot analysis using a polyclonal anticaspase 3 shows no cleavage of procaspase 3.

We then sought to investigate whether L-DNase II could be involved in HMA-induced apoptosis. Immunofluorescent

Figure 3 Detection of L-DNase II in HMA-induced apoptosis. **(a)** BHK cells were treated for 24 h with or without 40 μ M HMA (HMA and Ctl, respectively). After treatment, cells were fixed and immunostained with a polyclonal anti-L-DNase II antibody and DAPI. The arrow shows a nuclear labeling of the protein. **(b)** BHK cells were incubated with 40 μ M HMA for the indicated times. Then the cells were homogenized and analyzed by Western blot with a polyclonal anti-LEI antibody. A measure of 25 μ g of total protein was loaded per well. Molecular weight markers (MW, kDa) are indicated on the left. The different forms of LEI/L-DNase II are shown on the right

studies using an anti-L-DNase II antibody showed an increased labeling in treated cells (Figure 3a). In the apoptotic cells, the L-DNase II labeling could be mainly localized in the nucleus, suggesting the activation of this pathway. We took advantage of the apparent molecular weight difference between LEI (42 kDa) and L-DNase II (27 kDa) to verify endonuclease activation by Western blot (Figure 3b). Moreover, as cells seem to commit very early to apoptosis (Figure 2a), we studied LEI conversion after different exposure times to HMA with an anti-LEI antibody (Figure 3b). One can observe the progressive apparition of L-DNase II between 8 and 24 h. We measured the total DNase II-like activity after 8 h of HMA treatment. Total cell extracts were incubated with a plasmid whose degradation was followed at different times in optimal enzymatic conditions for DNase II (acidic pH cation-free). HMA-treated cells showed an enhanced activity compared to untreated cells (Figure 4). Plasmid degradation was suppressed with a polyclonal L-DNase II antibody (Figure 4), which was found to inhibit recombinant L-DNase II activity (not shown). In conclusion, the majority of DNase II activity that is increased after HMA treatment can be attributed to L-DNase II.

Taken together these results suggest that HMA treatment induces apoptosis and may recruit the L-DNase II pathway. Similar results were obtained in COS-7 and CHO cell lines when apoptosis was induced by HMA in the same conditions (not shown), indicating that the activation of this pathway instead of caspases might not be cell-type dependent.

Figure 4 L-DNase II activity is increased following HMA treatment. Total DNase II activity was followed by plasmid degradation, on a 1% agarose gel, at the indicated times with total extracts of BHK untreated (Ctl) or treated with HMA 40 μ M during 8 h (HMA) as described in Materials and Methods. In the upper panels, one can observe the major supercoiled form of the plasmid at initial time. This band will be converted into the relaxed form and then into a linear form that disappears with a smear. The supercoiled form of the plasmid disappears at 45 min in HMA-treated cells while it is still visible at 60 min in control cells. Moreover, the linear band intensity decreases more quickly in the treated cells. This shows a higher activity in HMA-treated cells. In the lower panel, the same experiment was achieved in the presence of a polyclonal anti-L-DNase II (anti-DNase II) or a rabbit nonimmune serum (nonimmune). DNase II activity is completely inhibited. SC: supercoiled; L: linear, R: relaxed

The L-DNase II pathway is activated during HMA-induced apoptosis in BHK cells

In order to prove the involvement of LEI/L-DNase II in HMA-induced apoptosis, we overexpressed LEI in BHK cells by transfection using a pREP10 vector containing porcine LEI cDNA. Transiently transfected cells expressed a high level of LEI, but in these conditions few cells survived after HMA treatment (not shown). We therefore selected stable transfected cells using hygromycin B, a lethal antibiotic to BHK wild-type cells. Two types of clones were established, the LEI clones ('L clones') and the control clones ('C clones') transfected with the empty pREP10. Overexpression of LEI was verified by RT-PCR (Figure 5a, upper panel), since the difference of expression was not clearly seen on Western blots (not shown). LEI band quantification in comparison with the GAPDH band showed a similar expression rate between LEI and GAPDH. The absence of LEI signal in control cells is because of the use of specific probes for porcine LEI. When L and C clones were exposed to HMA, a significant increase in cell death is seen in most of LEI-overexpressing clones (Figure 5a, lower panel). Western blot showed a more sustained presence of L-DNase II (27 kDa) in these cells (Figure 5b). Moreover, the cleavage of LEI into its intermediary form of 35 kDa¹⁶ at 8 h, which has not been observed in wild-type cells, supported a higher L-DNase II activation in the LEI-overexpressing clones. These results indicate that LEI overexpression in BHK cells decreases their survival rate, supporting the hypothesis that this pathway is implicated in HMA-induced apoptosis. However, one cannot exclude the possibility that overexpression of LEI may lead to a hypersensitivity of the cells to any apoptotic inducer. To

Figure 5 Activation by HMA of the LEI/L-DNase II pathway in BHK cells. (a) RT-PCR of different BHK clones stably transfected with empty pREP10 (C4 and C5) or pREP10 containing the porcine LEI cDNA (L6-2, L6-5, L6-24). LEI lane: control RT-PCR of LEI inserted into the pGEM plasmid. MW indicates the bands length (bp). The different clones were induced to die with 40 μ M HMA for 24 h. Cell survival was determined with the MTT test and expressed as a percentage of the not treated cells of the same clone. The results represent the mean of three different experiments. *, ** $P < 0.05$ compared with the clone C5 and C4 respectively. ***: $P < 0.001$ compared with the clone C5. (b) Western blot analysis of LEI-overexpressing clone (L6-2) treated by HMA. A measure of 25 μ g of total protein was loaded per well. The LEI lane shows LEI expressed by *E. coli* (100 ng). On the left are the different forms of LEI/L-DNase II: 42 kDa (LEI), 35 kDa (intermediary form), 27 kDa (L-DNase II). MW indicates the molecular weight markers (kDa)

investigate this point, we triggered apoptosis in BHK cells using etoposide, which involves the caspase pathway.^{20,14,46}

Etoposide induces L-DNase II-independent apoptosis in BHK cells

Anderson and Roberge⁴⁷ showed that exposure of BHK cells to etoposide, an inhibitor of topoisomerase II, impairs mitosis in these cells. However, no study was done on the induction of cell death by this agent in these cells. The exposure of BHK cells to 100 μ M etoposide for 3 h, followed by a recovery period of 24 h, induced cell death in about 20% of the cells (not shown). Cells displayed condensed chromatin and internucleosomal degradation of DNA (Figure 6a) in accordance with an apoptotic cell death. In contrast to the cell death induced by HMA, immunocytochemistry using an anti-L-DNase II antibody did not reveal any difference between control and etoposide-treated cells (Figure 6b). Moreover, Western blot confirmed that LEI was not transformed into L-DNase II (Figure 6c). These results suggest that this pathway is not activated by etoposide.

Figure 6 Etoposide induces apoptosis but not L-DNase II in BHK cells. **(a)** DNA gel of BHK cells treated or not with 100 μ M etoposide during 3 h followed by 24 h of recovery. DNA degradation shows a ladder on etoposide-treated cells (lane Eto), which is not seen in control cells (lane Ctl); MW indicates the molecular weight markers (pb). **(b)** L-DNase II immunocytochemistry of BHK cells treated (etoposide) or not (Ctl) with etoposide as described above. The nuclei were labeled with DAPI. **(c)** Western blot on control and etoposide-treated cells. No difference is seen between control and treated cells. The LEI lane is recombinant LEI used as control

Figure 7 Effect of LEI overexpression on etoposide-induced apoptosis in BHK cells. **(a)** BHK clones (control: C4 and C5; LEI overexpressing: L6-2, L6-5, L6-24) were treated with 100 μ M etoposide for 3 h followed by 24 h of recovery. The cell survival was measured with the MTT test. The results represent the mean of three different experiments. No significant difference between the control clones and the LEI clones is observed. **(b)** Overexpression of LEI in transiently transfected cells as studied by Western blot with a polyclonal anti-LEI antibody. LEI lane: LEI expressed by *E. coli* (100 ng), EV lane, cells transfected with empty pREP10; LV lane, cells transfected with pREP-LEI. MW indicates molecular weight markers (kDa). **(c)** BHK cells (WT) and cells transiently transfected with LEI (LV) or with the plasmid vector without insert (EV) were treated with 100 μ M etoposide for 3 h followed by 24 h of recovery. The cell survival was calculated by the MTT method. * P < 0.05 compared with WT. ** P < 0.001 compared with EV

We then tested the sensitivity of LEI-overexpressing cells to etoposide. Stably transfected cells were subjected to the MTT test after treatment. The MTT test showed no significant survival difference between clones containing the plasmid

carrying the LEI cDNA and the clones containing the empty vector (Figure 7a). This result is consistent with the lack of recruitment of L-DNase II by etoposide, as shown in immunocytochemistry experiments. However, if a high level of LEI expression by transient transfection is obtained (Figure 7b), LEI overexpressing cells treated with etoposide survived significantly better than cells transfected with the empty vector (Figure 7c). It is possible that LEI overexpression could have an effect on the cell cycle of BHK cells. In this way, a cell cycle modification rather than an LEI effect on apoptosis could mimic a protection by increasing the dividing cells. To address this possibility, survival rates were compared between untreated transfected cells with LEI cDNA to untreated cells transfected with the empty vector (ANOVA test followed by a *post hoc* analysis). The result ($P=0.34$) rules out this possibility, thus suggesting an antiapoptotic effect of LEI in etoposide-induced apoptosis.

The results on cell survival obtained by inducing apoptosis in LEI overexpressing cells with either HMA or etoposide indicate that LEI overexpression may either favor or impair apoptosis depending on the apoptotic inducer.

Discussion

L-DNase II is an endonuclease involved in internucleosomal degradation of nuclear DNA during apoptosis.^{9,11–13} Its precursor LEI belongs to the serpin superfamily and can be classified among the ovalbumin serpins.⁴⁸ Like most serpins, LEI has antiprotease activity. In its native form it inhibits elastase, cathepsin G⁴⁹ and probably other proteases. After post-translational modification, LEI loses its inhibitory activity and acquires endonuclease activity.¹⁶ We hypothesized that LEI/L-DNase II may play a critical role in cell survival, acting as a molecular switch that when activated can lead to apoptosis.²⁷ Normally, LEI may inhibit proteolytic activity. When converted into L-DNase II, however, LEI could release this proteolytic inhibition and instead promote nuclear degradation in apoptotic cells. The switch between LEI and L-DNase II may be catalyzed during apoptosis by elastase¹² or by acidic

proteases.³⁷ These later enzymes may be activated by a decrease in pH. Since pH decrease is frequently observed during apoptosis,^{20,28–32} we hypothesized that pH decrease may be a causative factor in the transition from LEI to L-DNase II.

Eucaryotic cells use a range of membrane transport mechanisms to regulate pH. The electroneutral Na⁺/H⁺ antiport, which exchanges intracellular H⁺ with extracellular Na⁺, is presumed to be present in all eucaryotic cells. Here we confirmed by RT-PCR that BHK cells express the NHE1 isoform of the Na⁺/H⁺ antiport and that NHE activity is sensitive to HMA. This finding is not surprising since NHE1 is considered to be the housekeeping isoform responsible for the maintenance of cytosolic pH and cellular volume.³⁸ The isoforms 2 and 3 were also detected and were already described in the kidney.⁵⁰ These results are supported by a previous report of NHE activity in BHK cells.⁵¹

Amiloride derivatives inhibit NHE and can induce intracellular acidification.³⁸ In BHK cells, NHE antiport is also sensitive to amiloride, as shown by acidification experiments. We therefore treated BHK cells with HMA to decrease intracellular pH. In the presence of HMA, most cells undergo apoptosis as shown by apoptotic-like DNA degradation and morphological changes. These results are in accordance with data from other groups showing apoptotic induction by HMA and other amiloride derivatives^{43,44} and intracellular acidification during apoptosis.^{33–35} Moreover, the obtained acidification with HMA is in agreement with that obtained by other groups using the same H⁺ antiport inhibitor.⁵²

HMA's proapoptotic effect was quite rapid. After only 1 h of treatment with HMA, more than 50% of cells were found to be apoptotic and 76% after 4 h of treatment (apoptosis was always measured 24 h after the beginning of treatment). This suggests that HMA acts very fast and that most of the cells cannot be saved from apoptosis after a short exposure to HMA. In addition, we showed that the L-DNase II pathway is recruited early during the HMA-induced apoptosis course. Involvement of the LEI/L-DNase II pathway in HMA-induced apoptosis was confirmed by overexpression experiments. Classically, the function of a protein is better studied by deletion (anti-sense, RNAi, knockout). However, as this protein has two distinct activities, the L-DNase II deletion implicates also the LEI deletion and therefore the addition of two distinct effects. If LEI, the L-DNase II precursor, is overexpressed, cell death is amplified two- to three-fold in BHK cells in the presence of HMA. However, it was important to check whether this amplification might be a nonspecific effect of LEI overexpression. We therefore also performed LEI overexpression experiments in the context of etoposide-induced apoptosis, since etoposide-induced apoptosis is not associated with LEI-to-L-DNase II transformation.¹⁴ In these experiments, stable LEI overexpression has no significant effect on BHK cell survival. However, transient LEI overexpression (high level of LEI) protects cells from etoposide-induced cell death. This result may indicate that in its native form LEI can present some antiapoptotic properties. Apoptosis-modulating functions have been reported for other serpins,^{19,21,53} although their effects are mixed. For instance, PI-9 inhibits apoptosis induced by granzyme B but not apoptosis induced by Fas ligand²¹ and maspin overexpres-

sion increases apoptosis.⁵⁴ It is widely known that etoposide induces apoptosis by activating the caspases pathway.^{20,14,46} Since diverse residues are implicated in target specificity of serpins⁵⁵ and although caspases do not possess known LEI-reactive sequences, some of these proteases may be found to be targets of LEI upon further understanding of LEI target specificity.

We have previously shown that L-DNase II is activated by several apoptotic inducers. Ethanol,¹³ staurosporine¹² and long-term culture¹⁴ can transform LEI into L-DNase II in cellular models. Interestingly, most of these agents can also induce intracellular acidification.^{28,30} In the present study, we showed that decreasing pH through inhibition of NHE causes the conversion of LEI into L-DNase II. The finding of the pH dependence of L-DNase II activation will contribute to a better understanding of L-DNase II activation in these models. Furthermore, a number of studies have shown that etoposide induces an intracellular acidification^{20,56,57} and only little data proved the contrary.⁵⁸ Since etoposide does not activate L-DNase II in BHK cells, this suggests that decrease of pH favors the transformation of LEI into L-DNase II but that specific factors may also participate. All the results presented in this manuscript and in previous studies about the dual activities of LEI/L-DNase II are summarized in Figure 8.

The LEI/L-DNase II pathway provides a novel example of important roles for protein cleavage signaling during apoptosis. Many cleavage targets are known: the inhibitor of caspase-activated DNase (ICAD), lamins, cytoskeletal proteins, PARP-1, p21-activated kinase-2, Rho-activated serine/threonine kinase.¹⁵ The survival functions of all of these proteins are suppressed upon their cleavage. Other proteins acquire an anti- or a proapoptotic role after processing. Proteolysis of presenilin-2, which is involved in Alzheimer's disease-associated cell death, liberates an antiapoptotic polypeptide and generates a negative feedback loop.⁵⁹ Caspases are produced as zymogen and acquire apoptotic activity after proteolytic modification.⁶⁰ In addition, several members of the Bcl-2 family acquire proapoptotic roles upon processing. Truncated BID transduces apoptotic signals by translocation to mitochondria.⁶¹ The same holds true for BAD and Bcl-x(L), which become potent proapoptotic agents after cleavage and translocation to the mitochondria membrane.^{62,63} Diverse proteins that present dual-function regulation of apoptosis are emerging. The procaspase 2 is subjected to alternative splicing, generating a short isoform able to inhibit etoposide-mediated apoptosis, while the long isoform participates in Fas-mediated apoptosis.⁶⁴ Depending on its protein partners, Bruton's tyrosine kinase promotes radiation-induced apoptosis, but inhibits Fas-activated apoptosis.⁶⁵ The 15 kDa phosphoprotein enriched in astrocytes (PEA-15) presents a bifunctional regulation similar to LEI/L-DNase II. Normally, it favors TNF-induced apoptosis while in its phosphorylated state it is a necessary factor in the inhibition of Fas-mediated apoptosis.⁶⁶ Here, we showed that the dual protein LEI/L-DNase II presents differential apoptosis-modulation effects in response to different inducers. As the PEA-15, this duality is permitted by a post-translational modification.

In conclusion, our study suggests that the LEI/L-DNase II pathway can participate in switching cells from survival to

Figure 8 Model of LEI/L-DNase II dual function in apoptosis. In living cells, LEI is the major form and L-DNase II is in a basal level of activity. Since LEI inhibits elastase and cathepsin G *in vitro*, it may act as an inhibitor of diverse proteases promoting cell survival. When the cell is subjected to an apoptotic stress that induces pH_i decrease, the conversion of LEI into L-DNase II is favored. This may liberate proteases from inhibition and promote endonuclease activation

apoptosis. We show that LEI is a proapoptotic factor in response to Na⁺/H⁺ exchanger inhibition because of its conversion into L-DNase II. This proapoptotic function is easily understandable. In some cases, LEI can present some antiapoptotic properties. Further investigation into the mechanisms of the antiapoptotic function of LEI are underway in our laboratory.

Materials and Methods

Materials

Etoposide was obtained from Alexis Corporation; low melting agarose, 4',6-diamidino-2-phenylindole (DAPI), HMA, tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5 phenyltetrazolium bromide (MTT) and RNase A were purchased from Sigma; proteinase K and DNA molecular weight markers for electrophoresis were from BioRad. Immobilon P was obtained from Millipore. TRITC-conjugated and peroxidase-conjugated goat anti-rabbit IgG were from Biosys. Polyclonal anticaspase 3 was from Santa Cruz Biotechnology. Polyclonal antibodies against L-DNase II and LEI were prepared in our laboratory.⁹ Anti-L-DNase II antibody was prepared using commercial DNase II from Worthington (reference 58P2465Y), which is identical to L-DNase II (as determined by protein sequencing).¹⁶ Anti-LEI antibody was prepared by using a synthetic peptide from region 181 to 196 from LEI. Oligos for PCR were synthesized by MWG. The expression vector pREP10 was from Invitrogen. Bafilomycin A1 and tetramethylammonium chloride (TMACl) were from Sigma, and nigericin and carboxyl-seminaphthorhodafluor-1 acetoxymethylester (SNARF-1) from Molecular Probes.

Cell culture and treatment

BHK cells were grown as monolayers in Dulbecco's modified Eagle's medium (D-MEM, Life Technology) supplemented with 10% fetal calf serum (FCS), 4 mM glutamine, 200 U/ml penicillin and 0.2 mg/ml streptomycin and 5 μ g/ml fungizone (all from Life Technology). Cells were grown at 37°C in a humidified atmosphere containing 5% CO₂.

BHK cells were seeded at a density of 20 000 cells/cm², maintained in culture for 2 days and then treated for 24 h with 40 μ M HMA. For etoposide treatment, the cells were incubated for 3 h with 100 μ M etoposide. Thereafter, the drug was removed and cells were maintained in culture with fresh medium for 24 h.

RT-PCR

Total RNA from 5 $\times$ 10⁵ BHK cells was purified using the Absolutely RNA RT-PCR Miniprep kit (Stratagene) according to the manufacturer's instructions. Total RNA extracted from rat kidney used as control was kindly provided by Dr. J Poggiali. A total of 2.5 μ g of RNA was retrotranscribed from a poly(dT) primer by reverse transcriptase from Moloney murine leukemia virus (Life Technology) and then amplified by PCR using rat NHE-specific primers.⁴⁰

The porcine LEI expression was checked by RT-PCR in stably transfected BHK cells. Total RNA was extracted with the RNeasy kit (Qiagen). A total of 2 μ g of RNA was used for RT-PCR as above. The primers used were: porcine LEI: sense 5'-CAC CTG ACC CTG GAA AAG C-3', position 751, antisense 5'-CGG ATG AAG AAA ATG AAC G-3', position 1073; hamster GAPDH: sense 5'-ATG CCC CCA TGT TTG TGA TG-3', position 344, antisense 5'-ATG GCA TGG ACT GTG GTC AT-3', position 487.

PCR products were loaded on 1% agarose gel and visualized by staining with ethidium bromide.

Flow cytometry

For pH measurements, cells were loaded for 30 min with 5 μ M carboxyl-SNARF-1 AM in a loading solution (110 mM NaCl, 30 mM TMACl, 3 mM KCl, 0.2 mM KH₂PO₄, 0.8 mM K₂HPO₄, 1 mM CaCl₂, 1 mM MgSO₄, 10 mM HEPES pH 7.4) and analyzed on a flow cytometer (EPICS Altra, Beckman-Coulter) with excitation at 488 nm and emission measured at 575 and 640 nm. Quantification of the number of cells in various populations was obtained by drawing regions on the profiles and excluding cells with fluorescence < 50 to avoid cells that either do not load with dye or that have lost membrane integrity. The pH measurements were obtained by rationing the fluorescence emissions (575/640) at the two appropriate wavelengths and generating a pH calibration curve.^{44,67}

Calibration of the signal was achieved by incubating SNARF-1-loaded BHK cells for 20 min at 37°C in the presence of 10 μ M nigericin in high potassium buffer at pH 6.0, 6.5, 7.0, 7.5 and 8.0.⁶⁸

To measure the activity of the Na⁺-H⁺ exchanger, pH_i was reduced using the NH₄⁺ pulse technique.⁴² Cells suspended were exposed 30 min to 40 mM NH₄Cl in a bicarbonate-free solution (140 mM TMACl, 3 mM KCl, 0.2 mM KH₂PO₄, 0.8 mM K₂HPO₄, 1 mM CaCl₂, 1 mM MgSO₄, 10 mM HEPES pH 7.4) in the presence of 5 μ M SNARF-1 and 40 nM bafilomycin A1. Then cells were centrifuged (10 000 $\times$ g C'est la petite boule, peut être enlever la paranthèse et mettre cells were briefly centrifuged, 15 s) and resuspended in the bicarbonate-free solution containing 40 nM bafilomycin A1. Recovery of pH_i in the presence of a 110 mM Na⁺ solution was recorded in the presence of 40 nM bafilomycin A1 and in the presence or absence of 40 μ M HMA.

Analysis of DNA fragmentation

Genomic DNA was analyzed using the Zhu and Wang⁶⁹ method. Cells were detached from the culture flask using a PBS-EDTA solution (19 mM Na₂HPO₄, 1 mM KH₂PO₄, 140 mM NaCl, 15 mM KCl, 5 mM EDTA pH 7.5). Cells (2×10^6) were disrupted with 120 μ l of the lysis buffer (50 mM Tris, EDTA 10 mM, sarkosyl 2%, pH 7.4). Samples were then incubated with 1 μ l of 20 mg/ml Proteinase K for 2 h at 45°C, and then 1 h at 37°C with 8 μ l of 1 mg/ml RNase A DNase free. From this mixture, 10 μ l was then loaded with 2 μ l of sample buffer (30% glycerol, 0.25% xylene blue, 0.25% bromophenol blue) onto a 1% agarose gel. Agarose horizontal slab gels were made up in 40 mM Tris-acetic acid, pH 7.8, 20 mM NaCl and 2 mM EDTA in the presence of ethidium bromide. The samples were loaded and run at 100 V until bromophenol blue reached 5 cm. Gels were then analyzed on a UV transilluminator (302 nm, Gel Doc) and photographed using a numeric camera.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 15 min, washed in PBS and permeabilized with 0.3% Triton X-100 in PBS for 30 min. After washing with PBS, cells were saturated for 30 min at room temperature with PBS containing 1% skim milk and then incubated for 1 h at room temperature with an anti-L-DNase II (1/100) in PBS-0.1% milk. This incubation was followed by five washes for 5 min in PBS-0.1% milk. The antibody was localized using a TRITC goat anti-rabbit IgG (1/500 dilution) in PBS-0.1% milk (1 h, room temperature). Cells were then washed five times for 5 min in PBS. During the last wash in PBS, nuclei were revealed with the fluorescent nuclear stain DAPI and then mounted with 50% glycerol in PBS. Results were evaluated under a Leitz Aristoplan microscope equipped with an epi-illuminator HBO and filters for rhodamine and DAPI fluorescence. They were photographed using a Spot II numeric camera.

Immunoblotting detection of L-DNase II

Protein samples for electrophoresis were prepared by mixing with Laemmli buffer. Gel electrophoresis was conducted at a constant current of 20 mA using 12% polyacrylamide slab gels. Transfer of proteins to Immobilon P membranes was carried out electrophoretically.⁷⁰ A constant voltage of 40 V was applied for 16 h. Immediately after blotting, the membranes were soaked for 2 h at 37°C in PBS containing 5% fat-free dried milk. Thereafter, the blot was incubated for 1 h at room temperature with an anti-L-DNase II diluted (1/1000) in PBS containing 0.1% Tween (PBS-Tween buffer). The binding of the antibodies was visualized after two washings (10 min each) with PBS-Tween buffer, followed by 1 h incubation with peroxidase-conjugated goat anti-rabbit IgG (1 μ g/ml) in PBS-Tween buffer. The sheets were then rinsed successively with PBS-Tween buffer (3 $\times$ 5 min) and finally with PBS (2 $\times$ 5 min). Peroxidase activity was detected using the ECL⁺ method (Amersham). No signal was detected with rabbit preimmune serum.

L-DNase activity assay

BHK cells were treated with 40 μ M HMA for 8 h. Then 3×10^7 cells were homogenized in ice, using a Potter homogenizer, in 1 ml of 10 mM Tris-HCl pH 7.4 containing 1 M NaCl, 1 mM PMSF, 5 mM phenanthroline, 1 μ g/ml leupeptin and 0.2 μ g/ml aprotinin. Extracts were then disrupted with a sonicator for 1 s three times. The homogenate was centrifuged (10 000 $\times$ g, 4°C) for 30 min. The supernatant was dialyzed overnight at 4°C against 10 mM Tris-HCl pH 7.4, 1 mM EDTA in the presence of

0.1 mM PMSF, 0.5 mM phenanthroline, 0.1 μ g/ml leupeptin and 20 ng/ml aprotinin. Protein concentration was evaluated with the BCA method (Pierce) using bovine serum albumin (BSA) as standard.

Total DNase II activity was measured by incubating 1 μ g of cell extract at 37°C in a final volume of 60 μ l of 10 mM Tris and 10 mM EDTA (pH 5.5) containing 2 μ g of plasmid DNA. Aliquots (10 μ l) were frozen at different incubation times and then loaded on a 1% agarose gel. L-DNase II activity was inhibited by a preincubation with a polyclonal anti-L-DNase II or rabbit nonimmune serum (1/10 of final volume) in the absence of plasmid for 1 h at 37°C. The reaction was then started by the addition of the plasmid.

Transient overexpression of LEI

To perform overexpression experiments, the porcine LEI cDNA was subcloned from pGEM¹⁶ into the *Xho*/*Nco*I restriction sites of pREP10 (Invitrogen). *E. coli* DH5 α strand was transformed by electroporation and plasmid DNA was prepared using the Qiafilter Plasmid Maxi kit.

BHK cells were seeded at a density of 20 000 cells/cm² and cultured as described for 48 h. At 2 h before transfection, the culture medium was replaced by fresh medium. The transfection medium containing 10 μ g of pREP-LEI and 60 μ l of Lipofectin reagent (Life Technologies, Inc.) in 0.5 ml of serum-free DMEM was incubated 20 min at room temperature, then diluted with DMEM to a final volume of 2.5 ml and added to BHK cells. The transfection process occurred at 37°C for 4 h, then 2.5 ml of DMEM containing 20% FCS serum was added to the cells. Control cells were prepared by using the pREP10 empty plasmid.

Stable overexpression of LEI

BHK cell sensitivity to hygromycin B was first tested by exposing the cells to 50–1000 μ g/ml hygromycin B. A concentration of 500 μ g/ml was finally chosen to select transfected cells. The cells obtained after transfection and a week survival in hygromycin B were then seeded into a 96-well culture plate at a clonal density. In total, 24 clones overexpressing LEI were obtained and named L6-1 to 24. Six control clones, containing the empty vector, were also obtained.

MTT reduction assay

BHK cells were seeded and treated, as described above, in a 24-well plate. The MTT was diluted in PBS at a concentration of 1 mg/ml. At the end of cell treatment, culture medium was removed and 250 μ l was added to each well. The plate was kept in a CO₂ incubator for 1 h. The cells were then lysed by the addition of a lysis solution (50% dimethylformamide, 20% SDS, pH 4.7). The degree of MTT reduction in each sample was subsequently assessed by measuring absorption at 570 nm using a microplate reader (BioRad).^{21,34} The survival rate was expressed as the percentage of the untreated cells.

Statistics

Results are presented as means $\pm$ S.E.M. Unless otherwise stated, statistical significance was assessed by Student's *t*-test.

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References

- Majno G and Joris I (1995) Apoptosis, oncosis, and necrosis. An overview of cell death. *Am. J. Pathol.* 146: 3–15
- Montague JW, Gaido ML, Frye C and Cidlowski JA (1994) A calcium-dependent nuclease from apoptotic rat thymocytes is homologous with cyclophilin. Recombinant cyclophilins A, B, and C have nuclease activity. *J. Biol. Chem.* 269: 18877–18880
- Peitsch MC, Polzar B, Stephan H, Crompton T, MacDonald HR, Mannherz HG and Tschoep J (1993) Characterization of the endogenous deoxyribonuclease involved in nuclear DNA degradation during apoptosis (programmed cell death). *EMBO J.* 12: 371–377
- Shiokawa D and Tanuma S (1998) Molecular cloning and expression of a cDNA encoding an apoptotic endonuclease DNase gamma. *Biochem. J.* 332: 713–720
- Pandey S, Walker PR and Sikorska M (1997) Identification of a novel 97 kDa endonuclease capable of internucleosomal DNA cleavage. *Biochemistry* 36: 711–720
- Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A and Nagata S (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* 391: 43–50
- Barry MA and Eastman A (1993) Identification of deoxyribonuclease II as an endonuclease involved in apoptosis. *Arch. Biochem. Biophys.* 300: 440–450
- Gottlieb RA, Giesing HA, Engler RL and Babior BM (1995) The acid deoxyribonuclease of neutrophils: a possible participant in apoptosis-associated genome destruction. *Blood* 86: 2414–2418
- Torrìglia A, Chaudun E, Chany-Fournier F, Jeanny JC, Courtois Y and Counis MF (1995) Involvement of DNase II in nuclear degeneration during lens cell differentiation. *J. Biol. Chem.* 270: 28579–28585
- Counis MF, Chaudun E, Arruti C, Oliver L, Sanwal M, Courtois Y and Torrìglia A (1998). Analysis of nuclear degradation during lens cell differentiation. *Cell Death Differ.* 5: 251–261
- Torrìglia A, Chaudun E, Chany-Fournier F, Courtois Y and Counis MF (2001) Involvement of L-DNase II in nuclear degeneration during chick retina development. *Exp. Eye Res.* 72: 443–453
- Belmokhtar CA, Torrìglia A, Counis MF, Courtois Y, Jacquemin-Sablon A and Segal-Bendirdjian E (2000) Nuclear translocation of a leukocyte elastase inhibitor/elastase complex during staurosporine-induced apoptosis: role in the generation of nuclear L-DNase II activity. *Exp. Cell Res.* 254: 99–109
- Brossas JY, Tanguy R, Perani P, Torrìglia A, Chaudun E, Counis MF, Courtois Y and Tréton, J (1999) A new cell death pathway in cultured human retinal pigmented epithelial cells. *Les Séminaires Ophtalmologiques d'IPSEN, Vision, Gène et Cerveau* 10: 119–130
- Torrìglia A, Negri C, Chaudun E, Prosperi E, Courtois Y, Counis MF and Scovassi AI (1999) Differential involvement of DNases in HeLa cell apoptosis induced by etoposide and long term-culture. *Cell Death Differ.* 6: 234–244
- Leist M and Jaattela M (2001) Four deaths and a funeral: from caspases to alternative mechanisms. *Nat. Rev. Mol. Cell Biol.* 2: 589–598
- Torrìglia A, Perani P, Brossas JY, Chaudun E, Treton J, Courtois Y and Counis MF (1998) L-DNase II, a molecule that links proteases and endonucleases in apoptosis, derives from the ubiquitous serpin leukocyte elastase inhibitor. *Mol. Cell Biol.* 18: 3612–3619
- Bird PI (1999) Regulation of pro-apoptotic leukocyte granule serine proteinases by intracellular serpins. *Immunol. Cell Biol.* 77: 47–57
- Cao Y (2001) Endogenous angiogenesis inhibitors and their therapeutic implications. *Int. J. Biochem. Cell Biol.* 33: 357–369
- Enari M, Hug H and Nagata S (1995) Involvement of an ICE-like protease in Fas-mediated apoptosis. *Nature* 375: 78–81
- Furlong IJ, Ascaso R, Lopez Rivas A and Collins MK (1997) Intracellular acidification induces apoptosis by stimulating ICE-like protease activity. *J. Cell Sci.* 110: 653–661
- Bird CH, Sutton VR, Sun J, Hirst CE, Novak A, Kumar S, Trapani JA and Bird PI (1998) Selective regulation of apoptosis: the cytotoxic lymphocyte serpin proteinase inhibitor 9 protects against granzyme B-mediated apoptosis without perturbing the Fas cell death pathway. *Mol. Cell Biol.* 18: 6387–6398
- Los M, Neubuser D, Coy JF, Mozoluk M, Poustka A and Schulze-Osthoff K (2000) Functional characterization of DNase X, a novel endonuclease expressed in muscle cells. *Biochemistry* 39: 7365–7373
- Mukae N, Enari M, Sakahira H, Fukuda Y, Inazawa J, Toh H and Nagata S (1998) Molecular cloning and characterization of human caspase-activated DNase. *Proc. Natl. Acad. Sci. USA* 95: 9123–9128
- Oliveri M, Daga A, Cantoni C, Lunardi C, Millo R and Puccetti A (2001) DNase I mediates internucleosomal DNA degradation in human cells undergoing drug-induced apoptosis. *Eur. J. Immunol.* 31: 743–751
- Polzar B, Peitsch MC, Loos R, Tschoep J and Mannherz HG (1993) Overexpression of deoxyribonuclease I (DNase I) transfected into COS-cells: its distribution during apoptotic cell death. *Eur. J. Cell Biol.* 62: 397–405
- Krieser RJ and Eastman A (1998) The cloning and expression of human deoxyribonuclease II. A possible role in apoptosis. *J. Biol. Chem.* 273: 30909–30914
- Torrìglia A, Perani P and Courtois Y (1999) L-DNase II: a new molecule in apoptosis pathways. *Med. Sci.* 15: 253–259
- Matsuyama S, Llopis J, Deveraux QL, Tsien RY and Reed JC (2000) Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. *Nat. Cell Biol.* 2: 318–325
- Liu D, Martino G, Thangaraju M, Sharma M, Halwani F, Shen SH, Patel YC and Srikant CB (2000) Caspase-8-mediated intracellular acidification precedes mitochondrial dysfunction in somatostatin-induced apoptosis. *J. Biol. Chem.* 275: 9244–9250
- Thangaraju M, Sharma K, Leber B, Andrews DW, Shen SH and Srikant CB (1999) Regulation of acidification and apoptosis by SHP-1 and Bcl-2. *J. Biol. Chem.* 274: 29549–29557
- Rebollo A, Gomez J, Martinez de Aragon A, Lastres P, Silva A and Perez-Sala D (1995) Apoptosis induced by IL-2 withdrawal is associated with an intracellular acidification. *Exp. Cell Res.* 218: 581–585
- Petit PX, Gendron MC, Schrantz N, Metivier D, Kroemer G, Maciorowska Z, Sureau F and Koester S (2001) Oxidation of pyridine nucleotides during Fas- and ceramide-induced apoptosis in Jurkat cells: correlation with changes in mitochondria, glutathione depletion, intracellular acidification and caspase 3 activation. *Biochem. J.* 353: 357–367
- Perez-Sala D, Collado-Escobar D and Mollinedo F (1995) Intracellular alkalization suppresses lovastatin-induced apoptosis in HL-60 cells through the inactivation of a pH-dependent endonuclease. *J. Biol. Chem.* 270: 6235–6242
- Sharma K and Srikant CB (1998) Induction of wild-type p53, Bax, and acidic endonuclease during somatostatin-induced apoptosis in MCF-7 human breast cancer cells. *Int. J. Cancer* 76: 259–266
- Yanagisawa-Shiota F, Sakagami H, Kuribayashi N, Iida M, Sakagami T and Takeda M (1995) Endonuclease activity and induction of DNA fragmentation in human myelogenous leukemic cell lines. *Anticancer Res.* 15: 259–265
- Torrìglia A, Perani P, Brossas JY, Altairac S, Zeggai S, Martin E, Treton J, Courtois Y and Counis MF (2000) A caspase-independent cell clearance program. The LEI/L-DNase II pathway. *Ann. NY Acad. Sci.* 926: 192–203
- Altairac S, Chaudun E, Courtois Y and Torrìglia A (2001) Elastase is not required for L-DNase II activation during apoptosis in developing chicken neural retina. *Neurosci. Lett.* 303: 41–44
- Orlowski J and Grinstein S (1997) Na⁺/H⁺ exchangers of mammalian cells. *J. Biol. Chem.* 272: 22373–22376
- Counillon L and Pouyssegur J (1993) Nucleotide sequence of the Chinese hamster Na⁺/H⁺ exchanger NHE1. *Biochim. Biophys. Acta* 1172: 343–345
- Borensztein P, Froissart M, Laghmani K, Bichara M and Paillard M (1995) RT-PCR analysis of Na⁺/H⁺ exchanger mRNAs in rat medullary thick ascending limb. *Am. J. Physiol.* 268: F1224–F1228
- Dyck JR, Silva NL and Fiegel L (1995) Activation of the Na⁺/H⁺ exchanger gene by the transcription factor AP-2. *J. Biol. Chem.* 270: 1375–1381
- Roos A and Boron WF (1981) Intracellular pH. *Physiol. Rev.* 61: 296–434
- Park HJ, Makepeace CM, Lyons JC and Song CW (1996) Effect of intracellular acidity and ionomycin on apoptosis in HL-60 cells. *Eur. J. Cancer* 32A: 540–546
- Rich IN, Worthington-White D, Garden OA and Musk P (2000) Apoptosis of leukemic cells accompanies reduction in intracellular pH after targeted inhibition of the Na⁽⁺⁾/H⁽⁺⁾ exchanger. *Blood* 95: 1427–1434

45. Boyle KM, Irwin JP, Humes BR and Runge SW (1997) Apoptosis in C3H-10T1/2 cells: roles of intracellular pH, protein kinase C, and the Na⁺/H⁺ antiporter. *J. Cell Biochem.* 67: 231–240
46. Amarante-Mendes GP, Finucane DM, Martin SJ, Cotter TG, Salvesen GS and Green DR (1998) Anti-apoptotic oncogenes prevent caspase-dependent and independent commitment for cell death. *Cell Death Diff.* 5: 298–306
47. Anderson H and Roberge M (1996) Topoisomerase II inhibitors affect entry into mitosis and chromosome condensation in BHK cells. *Cell Growth Diff.* 7: 83–90
48. Korpula-Mastalerz R and Dubin A (1996) The intracellular serpin family. *Acta Biochim. Polon* 43: 419–429
49. Potempa J, Dubin A, Watorek W and Travis J (1988) An elastase inhibitor from equine leukocyte cytosol belongs to the serpin superfamily. Further characterization and amino acid sequence of the reactive center. *J. Biol. Chem.* 263: 7364–7369
50. Orlowski J, Kandasamy RA and Shull GE (1992) Molecular cloning of putative members of the Na/H exchanger gene family. cDNA cloning, deduced amino acid sequence, and mRNA tissue expression of the rat Na/H exchanger NHE-1 and two structurally related proteins. *J. Biol. Chem.* 267: 9331–9339
51. Viniegra S, Cragoe EJ and Rabito CA (1992) Heterogeneity of the Na⁺/H⁺ antiport systems in renal cells. *Biochim. Biophys. Acta* 1106: 99–109
52. Marches R, Vitetta ES and Uhr JW (2001) A role for intracellular pH in membrane IgM-mediated cell death of human B lymphomas. *Proc. Natl. Acad. Sci. USA* 98: 3434–3439
53. Dickinson JL, Norris BJ, Jensen PH and Antalis TM (1998) The C-D interhelical domain of the serpin plasminogen activator inhibitor-type 2 is required for protection from TNF- α induced apoptosis. *Cell Death Diff.* 5: 163–171
54. Zhang M, Shi Y, Magit D, Furth PA and Sager R (2000) Reduced mammary tumor progression in WAP-TAg/WAP-maspin bitransgenic mice. *Oncogene* 19: 6053–6058
55. Gils A and Declercq PJ (1998) Structure–function relationships in serpins: current concepts and controversies. *Thromb. Haemostasis* 80: 531–541
56. Anthony ML, Zhao M and Brindle KM (1999) Inhibition of phosphatidylcholine biosynthesis following induction of apoptosis in HL-60 cells. *J. Biol. Chem.* 274: 19686–19692
57. Reynolds JE, Li J and Eastman A (1996) Detection of apoptosis by flow cytometry of cells simultaneously stained for intracellular pH (carboxy SNARF-1) and membrane permeability (Hoechst 33342). *Cytometry* 25: 349–357
58. Benson RS, Dive C and Watson AJ (1999) Cytoplasmic acidification is not an effector mechanism of VP16 or DEX-induced apoptosis in CEM T leukaemia cells. *J. Cell Sci.* 112: 1755–1760
59. Vito P, Ghayur T and D'Adamio L (1997) Generation of anti-apoptotic presenilin-2 polypeptides by alternative transcription, proteolysis, and caspase-3 cleavage. *J. Biol. Chem.* 272: 28315–28320
60. Nicholson DW and Thornberry NA (1997) Caspases: killer proteases. *Trends Biochem. Sci.* 22: 299–306
61. Li H, Zhu H, Xu CJ and Yuan J (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 94: 491–501
62. Basanez G, Zhang J, Chau BN, Maksiav G, Frolov VA, Brandt TA, Burch J, Hardwick JM and Zimmerberg J (2001) Pro-apoptotic cleavage products of Bcl-xL form cytochrome c-conducting pores in pure lipid membranes. *J. Biol. Chem.* 276: 31083–31091
63. Condorelli F, Salomoni P, Cotteret S, Cesi V, Srinivasula SM, Alnemri ES and Calabretta B (2001) Caspase cleavage enhances the apoptosis-inducing effects of BAD. *Mol. Cell Biol.* 21: 3025–3036
64. Droin N, Rebe C, Hammann A, Bertrand R and Solary E (2001) Modulation of apoptosis by procaspase-2 short isoform: selective inhibition of chromatin condensation, apoptotic body formation and phosphatidylserine externalization. *Oncogene* 20: 260–269
65. Uckun FM (1998) Bruton's tyrosine kinase (BTK) as a dual-function regulator of apoptosis. *Biochem. Pharmacol.* 56: 683–691
66. Estelles A, Charlton CA and Blau HM (1999) The phosphoprotein protein PEA-15 inhibits Fas- but increases TNF-R1-mediated caspase-8 activity and apoptosis. *Dev. Biol.* 216: 16–28
67. Li J and Eastman A (1995) Apoptosis in an interleukin-2-dependent cytotoxic T lymphocyte cell line is associated with intracellular acidification. Role of the Na⁺/H⁺ antiporter. *J. Biol. Chem.* 270: 3203–3211
68. Eastman A (1995) Assays for DNA fragmentation, endonucleases, and intracellular pH and Ca²⁺ associated with apoptosis. *Methods Cell Biol.* 46: 41–55
69. Zhu N and Wang Z (1997) An assay for DNA fragmentation in apoptosis without phenol/chloroform extraction and ethanol precipitation. *Anal. Biochem.* 246: 155–158
70. Towbin H, Staehelin T and Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76: 4350–4354
71. Liu Y, Peterson DA, Kimura H and Schubert D (1997) Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *J. Neurochem.* 69: 581–593