Activation of a low pH-dependent nuclease by apoptotic agents

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Abstract

Intracellular acidification caused by agents such as UV(C), etoposide or ceramide accompanies the progression of apoptosis. It is suggested that cellular acidosis may set favorable conditions for a dormant, low pH-dependent (acidic) nuclease, which could be involved in intranucleosomal genome degradation, a hallmark of programmed cell death. Here we show that exposure of HL-60 cells to acidotic/apoptotic agents results in the several-fold activation of a novel low pH-dependent (acidic) nuclease activity, as revealed by zymography. Its activity, which resides in nuclei, is associated with four polypeptides with apparent M_r of 56, 48, 45 and 40 kDa. Treatment of HeLa cells with UV(C) or ceramide causes also the up-regulation of an acidic nuclease activity which is represented by 70 and 62 kDa polypeptides. These observations suggest that acidic nuclease activation can be induced by the same apoptotic agents in different cell types. In HL-60 cells, acidic nuclease up-regulation triggered by acidotic agents follows the induction of AP-1 transcription factor active complexes and accompanies the progression of apoptosis. Inhibition of AP-1 factor activity caused by either anti-caspase/anti-acidotic agent Zn^{2+} or curcumin, an inhibitor of AP-1 binding to DNA and *c-jun* synthesis, protects cells from genome destruction. Acidic nuclease activation, however, is only partially inhibited by these factors. We propose that (i) the up-regulation of an acidic nuclease activity is governed by a regulatory pathway different from that responsible for AP-1 factor induction, caspases activation and intracellular acidification, and (ii) activation of an acidic nuclease does not cause any deleterious effects when AP-1 transcription factor induction, caspases activation and intracellular acidification are down-regulated. Thus, the acidic nuclease up-regulation alone is not a sufficient prerequisite for apoptosis.

Keywords: apoptosis; AP-1 transcription factor; ceramide; etoposide; low pH-dependent (acidic) nuclease; UV(C)

Abbreviations: EMSA, electrophoretic mobility shift assay; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate buffered saline

Introduction

Evidence that intracellular acidification is associated with the progression of apoptosis has been steadily accumulating. UV(C) irradiation,¹ Fas receptor activation,¹ treatment with ceramide¹ or etoposide,² growth factors withdrawal^{3,4} or protein kinase C inhibition⁵ all result in cellular acidosis, genome degradation and ensuing cell death. Inhibitors of isoprenylation, Na^+/H^+ antiporter-mediated cytoplasmic pH homeostasis,⁶ V-type ATPase-catalyzed H^+ transport⁷ or cystic fibrosis transmembrane regulator⁸ further complement the already impressive list of acidotic/apoptotic factors. It has been found that inhibition of apoptosis protects the genome from degradation and is accompanied by restoration of cytoplasmic pH to normal values. The range of factors, which protect cells from death and alleviate cellular acidosis, suggests that the onset of cellular acidification and apoptosis is controlled by intricate signal transduction pathway(s). Inhibition of apoptosis by reintroduction of growth factors, administration of PKC activators,^{6,9,10} overexpression of Bcl-2^{11,12} or Ha-ras¹³ suggest that cell survival depends strongly on pathways involving kinases Raf-1 and PKC. Direct quenching of the elevated cytoplasmic H^+ level also protects the genome from degradation and arrests cell death.¹ The presence of Zn^{2+} , inhibitors of ICE/CED3 death protease family^{12,14,15} or inhibitors of protein phosphatases¹⁶ all inhibit cellular acidification associated with programmed cell death. These observations suggest that cellular acidosis is regulated by upstream effectors such as phosphatases and caspases.

Acidotic/apoptotic agents such as UV(C) and ceramide^{17,18} or growth factor withdrawal¹⁹ also induce the formation of active AP-1 transcription factor complexes. It is thought that AP-1 dimer or one of its components (e.g. *c-jun*) may be responsible for transcription regulation of genes involved in programmed cell death.^{17,18,20–25} Hence, it is conceivable that cellular acidification and AP-1 induction are governed by a common upstream acting element that regulates the procession of events participating in programmed cell death. Despite of the accumulated evidence pertaining to the importance of intracellular acidification in programmed cell death, there is no direct evidence for the involvement of a low pH-dependent nuclease in this process. It has been postulated^{26–30} that decreased cellular pH sets permissive conditions for the manifestation of the activity of otherwise dormant DNase II like pH-dependent nuclease. Candidate pH-dependent apoptotic nucleases can be divided into two classes distinguished by

lower 30–37 kDa^{27,30–33} or higher 56–70 kDa M_r.^{34–36} The diversity of acidic nuclease forms may reflect tissue specificity, cell differentiation and, perhaps, different modes of post-translational modification. These forms were identified by the common property of activation by low pH *in vitro*, rather than by their direct involvement in apoptosis.

The relationship between intracellular acidification, AP-1 factor induction and acidic nuclease activity has not yet been determined. We hypothesize that factor(s) responsible for acidification and the induction of AP-1 transcription factor may up-regulate the acidic nuclease activity *in vivo* and this effect should still be observed *in vitro*. In this report we investigated the effect of UV(C), etoposide and ceramide treatment of HL-60 cells on the activity of a low pH-dependent nuclease. We found that these acidotic/apoptotic agents activate by several-fold an acidic nuclease which is different from DNase II. This activation followed the induction of AP-1 transcription factor and was accompanied by genome degradation. Moreover, using inhibitors of cellular acidification or AP-1 DNA-binding activity we established that the up-regulation of the acidic nuclease activity takes place independently of AP-1 induction and intracellular acidification. Possible biological relevance of cellular acidification, AP-1 transcription factor induction and acidic nuclease up-regulation is discussed.

Results

The activation of a low-pH dependent nuclease by U(V)C coincides with the progression of apoptosis

Since UV radiation induces intracellular acidification¹ and apoptosis^{1,37,38} in Jurkat and HL-60 cells, we decided to investigate the relationship between the UV(C)-evoked apoptosis and the activity of a low pH-dependent (acidic) nuclease. The activity of an acidic nuclease present in nuclear extracts of cells undergoing apoptosis was analyzed by zymography. Low levels of acidic nuclease activity were observed in nuclear extracts isolated from untreated HL-60 cells (Figure 1, left panel, lane C).

By 2 h post irradiation nuclease activity was readily detected. At 4 h post irradiation nuclease activity had increased reaching a maximal level at 8 h post treatment (Figure 1, left panel, lanes 4 and 8). Based on FACS analysis 36% of cells had degraded DNA (as indicated by subdiploid DNA content) 4 h post UV(C) and this number increased to 50% during next 4 h (Table 1). Together, these observations suggest that nuclease activation accompanies genome degradation. The acidic nuclease, detected in HL-60 cells upon activation by U(V)C irradiation, is represented by four polypeptides of apparent molecular weight (M_r) 56, 48, 45 and 40 kDa, as revealed by SDS–PAGE (Figure 1). The intensity of bands, which

Figure 1 Nucleolytic activities in nuclear extracts isolated from UV(C)-irradiated HL-60 cells. Left panel: zymography carried out at pH 5.2. Lane S, molecular weight standards; lane C, extract from unirradiated cells; lanes 1–8 h post irradiation. Right panel: digestion of calf thymus DNA (for details see Materials and Methods). The means $\pm$ S.E. of three experiments are presented

Table 1 UV(C), etoposide and ceramide-induced apoptosis in HL-60 cells

Hours	UV (C)	UV (C)+Zn	Etoposide	Etoposide+Zn	Ceramide	Ceramide+curcumin
1	1.2 $\pm$ 0.2	0.6 $\pm$ 0.05	0.8 $\pm$ 0.3	0.8 $\pm$ 0.1	4.5 $\pm$ 1.1	1.2 $\pm$ 0.2
2	6.8 $\pm$ 2.1	0.5 $\pm$ 0.3	3.5 $\pm$ 0.8	0.8 $\pm$ 0.3	11.2 $\pm$ 3.1	1.8 $\pm$ 0.5
4	36.7 $\pm$ 8.1	1.0 $\pm$ 0.5	30.6 $\pm$ 5.2	0.4 $\pm$ 0.2	46.2 $\pm$ 7.5	1.6 $\pm$ 0.3
8	50.6 $\pm$ 3.9	2.3 $\pm$ 1.3	43.4 $\pm$ 4.3	2.9 $\pm$ 1.1	70.1 $\pm$ 8.2	5.3 $\pm$ 1.2

The extent of apoptosis is shown as the percentage of cells that exhibit subdiploid DNA content. Mean values of at least three experiments are shown. The percentage of control cells with subdiploid DNA content was less than 1%

appeared on zymographs, was time-, temperature- and pH-dependent. Bands became visible when gels were incubated for 16–22 h at acidic pH but not at pH 7.5, regardless of the presence or absence of Ca and Mg ions. Increase in the incubation temperature from 20–37°C substantially accelerated bands appearance at low pH but not at high pH. Nucleolytic activity that was associated with 56–40 kDa bands has been also verified by autoradiography. On the basis of these observations, we conclude that 56, 48, 45 and 40 kDa polypeptides represent the acidic nuclease activity rather than different DNA-binding proteins. As this nuclease differs in both immunological properties³⁹ (Famulski *et al*, manuscript in preparation) and size from the well known acidic nuclease – DNase II,⁴⁰ we postulate that the nuclease monitored here represents a novel enzyme. It has been shown that in HL-60 cells an acidic nuclease but not a $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent nuclease is associated with the majority of the nucleolytic activity.³⁸ To confirm that, we measured deoxyribonuclease activities present in nuclear extracts. To this end, extracts obtained from UV(C)-treated cells were tested for their propensity to digest high molecular weight calf thymus DNA and the reaction product i.e. acid-soluble oligonucleotides were detected by spectrophotometry. Figure 1 (right panel) shows that UV(C) irradiation caused up to a 6-fold increase in the low pH-dependent (pH 5.2) nucleolytic activity. No significant changes in DNA digestion were found, however, when the same extracts were tested under conditions of higher pH (7.5) and in the presence of Ca and Mg ions. This means that the acidic nuclease rather than $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent nuclease activity is up-regulated by UV(C) in HL-60 cells.

The effect of Zn^{2+} on UV(C) or etoposide-evoked AP-1 induction, apoptosis and acidic nuclease activation

AP-1 transcription factor has been shown to have multiple functions in cellular processes such as proliferation, differentiation and apoptosis.⁴¹ As we noted before, factors that cause cellular acidosis are also known for their propensity to induce AP-1. If intracellular acidification, AP-1 induction and nuclease activation are controlled during apoptosis by a common upstream regulator, an inhibitor of apoptosis should down-regulate the formation of AP-1 active complexes, prevent genome destruction and counteract the activation of the acidic nuclease. Hence, we examined the possibility that the appearance of low pH-dependent nuclease active forms may be related to the indication of active AP-1 complexes. Nuclear extracts obtained from the UV(C) or etoposide-treated HL-60 cells were analyzed for the presence of AP-1 by electrophoretic mobility shift assay (EMSA), using a radioactively labeled double-stranded oligonucleotide containing the AP-1 binding sequence as a probe. As expected, the EMSA showed that UV(C) irradiation caused the induction of active AP-1 complexes (Figure 2, left panel).

Densitometric analysis of EMSA autoradiographs indicated that 39% of maximal induction took place during the first hour. The peak of induction (100%) was observed within 4 h and was followed by a 4-fold decrease at 8 h post-irradiation. Three independent experiments gave similar results. Exposure of cells to etoposide invariably led to maximal induction of AP-1 within 2 h (Figure 3, left panel) as was observed in four independent experiments.

Figure 2 Induction of AP-1 and activation of a low pH-dependent nuclease by UV(C). Left panel: electrophoretic mobility shift assay of AP-1. Lane C, control; lanes 1–8 h post irradiation. Right panel: zymography carried out at pH 5.2. Lanes 1–8 h post irradiation

Figure 3 Induction of AP-1 and activation of a low pH-dependent nuclease by etoposide. Left panel: electrophoretic mobility shift assay of AP-1. Lane C, control; lanes 1–8 h post etoposide treatment. Right panel: zymography carried out at pH 5.2. Lanes 1–8 h post etoposide treatment

It has been reported that Zn²⁺ administration protects cells from UV or etoposide-evoked apoptosis^{14,37} and prevents intracellular acidification¹⁴ by inhibiting caspase 3 activation.⁴² In this study we examined the effect of Zn²⁺ administration on the formation of active AP-1 complexes and progression of apoptosis in UV(C) or etoposide-treated HL-60 cells. Treatment of UV(C) irradiated cells with Zn²⁺ resulted in the inhibition of AP-1 binding activity (Figure 2, left panel), with only 17% of maximal activity observed during the first hour, 4% during the next hour and no binding noted after 4 h post irradiation. The presence of Zn²⁺ effectively countered etoposide-evoked formation of active AP-1 complexes, with only 5% of maximal DNA binding activity observed within 2 h post-etoposide and no activity detected after 4 h (Figure 3, left panel).

Cells displaying subdiploid DNA content were apparent during 2 h post UV(C) or post etoposide treatment. However, number of these cells did not exceed 7% of the total population (Table 1). Intracellular DNA degradation accelerated during next 2 h (30–36%) and became a prominent feature of the cultures after 8 h, when 40–50% of cells displayed subdiploid DNA content. In the presence of Zn ions, however, the number of cells with subdiploid DNA content was less than 3% at 8 h post treatment regardless of the apoptotic agent used. These data suggest that UV(C) or etoposide-induced formation of active AP-1 complexes preceded DNA degradation, while Zn²⁺ administration prevented the activation of AP-1 transcription factor and protected HL-60 cells from apoptosis.

Much to our surprise, however, administration of Zn ions did not fully inhibit the acidic nuclease activation caused by

UV(C). As shown in Figure 2 (right panel) Zn²⁺ only partially inhibited nuclease up-regulation during first 4 h post irradiation and had no effect on the nuclease activity after 8 h post treatment. Exposure of cells to etoposide also resulted in the activation of a low pH-dependent nuclease and the kinetics of the nuclease activity up-regulation was similar to that observed for UV(C) (Figure 3, right panel). Acidic nuclease activity, however, remained high even after administration of Zn²⁺ and rescue from apoptosis. Of note, no up-regulation of the Ca²⁺/Mg²⁺-dependent nucleolytic activity was detected in etoposide-treated cells by either zymography or the direct measurement of DNA digestion. Analysis of the data presented above led us to the following conclusions (i) acidotic/apoptotic agents induce the formation of AP-1 active complexes and up-regulate acidic nuclease activity, (ii) the appearance of these complexes precedes genome degradation, (iii) nuclease activity up-regulation follows the induction of AP-1 but is still increased even after AP-1 de-regulation and (iv) Zn²⁺ ions inhibit AP-1 induction and apoptosis but not the activation of a low pH-dependent nuclease. Hence AP-1 and caspase 3 activation is not required for the nuclease up-regulation.

The effect of exogenous ceramide on AP-1 induction, apoptosis and acidic nuclease activation

Ceramide is thought to be a key signaling molecule involved in the regulation of apoptotic and stress-related pathways.^{43,44} Since the apoptotic action of ceramide in HL-60 cells has been well documented,^{10,18} we examined its impact on the activity of a low pH-dependent nuclease *in vivo*, in conjunction

with AP-1 induction. Figure 4 (left panel) shows that the continuous exposure of HL-60 cells to exogenous ceramide caused a rapid and sustained induction of active AP-1 complexes.

Maximal induction was already observed within 1 h and the number of active complexes declined only to 66 and 64% after 2 and 4 h, respectively. A substantial number of active AP-1 complexes (33% of the maximal level) were still present after 8 h of ceramide treatment. This observation confirms the kinetics of AP-1 induction by ceramide that have been recently reported by others.¹⁸ Curcumin, a pigment derived from the spice tumeric, was reported to counteract ceramide-evoked AP-1 activation and apoptosis in HL-60 cells,¹⁸ probably due to a direct interaction of this pigment with AP-1 binding to its DNA binding motif.⁴⁵ We confirmed that the administration of curcumin strongly inhibits the activity of AP-1 in ceramide-treated cells (Figure 4, left panel). Only 11 and 9% of maximal DNA-binding activity was observed within 1 and 2 h respectively and no such activity was observed after 4 and 8 h.

Ceramide rapidly induced genome degradation, which is consistent with its role as an apoptotic second messenger. After 2 h, 11% of ceramide-treated cells displayed subdiploid DNA content. This subpopulation increased to 46% during the next 2 h and to 70% of cells were affected after 8 h (Table 1). Pretreatment of cells with curcumin rescued them from ceramide-evoked apoptosis (Table 1). It has been reported,⁴⁶ however, that prolonged incubation of HL-60 cells with curcumin, in the absence of serum factors, leads to cell death. In our hands, curcumin alone did not cause deleterious effects in HL-60 cells within the time frame studied (5% subdiploid cell count after 8 h).

We found, that ceramide is also a potent activator of the acidic nuclease *in vivo*. Figure 4 (right panel) shows appreciable activity of a low pH-dependent nuclease was observed during the first 2 h of ceramide treatment. This activity increased steadily during the next 6 h. The kinetics of nuclease activation by ceramide was very similar to that observed for genome degradation. We did not find, however that the $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent nucleolytic activity was up-regulated by ceramide administration. Curcumin, which counteracted ceramide-evoked apoptosis, strongly inhibited nuclease activity within the first 4 h but by 8 h was much less effective. The time course of curcumin down-regulation of nuclease activity in ceramide-treated cells may suggest that curcumin offsets rather than inhibits nuclease activation. Similar results were obtained when curcumin-pretreated cell cultures were exposed to UV(C) irradiation. AP-1 activity was greatly diminished while nuclease activation was inhibited only during first 4 h post treatment (not shown).

Activation of a low pH-dependent nuclease in HeLa cells

In Figures 1–4 we documented that three acidotic/apoptotic agents namely, UV(C), etoposide and ceramide caused the activation of a novel acidic nuclease in HL-60 cells (Figures 1–4). Next we asked whether this phenomenon is limited to HL-60 cells or can be demonstrated in other cell types. We therefore repeated these experiments using near confluent cultures of HeLa cells treated with apoptotic agents. Total cell extracts were obtained and analyzed by zymography after 4 h.

Figure 4 Induction of AP-1 and activation of a low pH-dependent nuclease by ceramide. Left panel: electrophoretic mobility shift assay of AP-1. Lane C, control; lanes 1–8 h of incubation with ceramide. Right panel: zymography carried out at pH 5.2. Lanes 1–8 h of incubation with ceramide

Figure 5 Zymography of an acidic nuclease present in total extracts obtained from UV(C) or ceramide-treated HeLa cells. Lane S, molecular weight standards; lane C, control extract; lane 1, 4 h after UV(C) irradiation; lane 2, 4 h after serum withdrawal; lane 3, 4 h of incubation with ceramide; lane 4, ceramide and Zn^{2+} coincubation; lane 5, ceramide and curcumin; lane 6, 4 h of incubation with Zn^{2+} after UV(C) irradiation

Untreated HeLa cells exhibited low activity of the acidic nuclease which was represented by the 62 kDa polypeptide (Figure 5, lane C). Serum withdrawal or UV(C) irradiation resulted in the substantial activation of this nuclease and caused the appearance of a novel 70 kDa form (Figure 5, lanes 1 and 2). Administration of ceramide caused the most efficient activation of the nucleolytic activity represented by the 62 and 70 kDa polypeptides (Figure 5, lane 3). Similar results were obtained when nuclear extracts, isolated from treated HeLa cells, were examined. Pre-treatment of HeLa cells with curcumin strongly inhibited the ceramide-evoked activation of both polypeptides (Figure 5, lane 5), whereas the administration of Zn^{2+} was much less effective (Figure 5, lane 4). Nuclease activation, caused by UV(C) irradiation was only partially alleviated by Zn ions (Figure 5, lane 6). This experiment clearly demonstrated that HeLa cells possess a low pH-dependent nuclease whose activity is up-regulated by acidotic/apoptotic agents and can be, at least partially, down-regulated by anti-apoptotic agents.

Discussion

Our study sought to establish the relationship between the progression of apoptosis and the regulation of a low pH-dependent (acidic) nuclease. Apoptosis is a natural mechanism whereby unwanted, sick or DNA-damaged cells are eliminated.⁴⁷ Ordained destruction of the genome, which is manifested by the intranucleosomal cleavage of chromatin, is considered a hallmark of apoptosis. The resulting DNA ladder is generally believed to result from activation of endogenous nuclease(s).⁴⁸ Apoptotic nucleases are expected to fulfill certain criteria. For example a candidate enzyme must be dormant or absent in living cells. After the initiation of apoptosis, its activity should increase either in response to altered intracellular milieu (e.g. increased intracellular Ca^{2+} level), increased protein expression or post-translational modifications.^{49,50} Activation of certain nucleases in response to the initiation of apoptosis was in fact observed during T cell receptor-mediated cell death of T cell

hybridomas and thymocytes,⁵¹ interleukin-2 deprivation of cytotoxic T-lymphocytes,⁵² administration of angiotensin II to ventricular myocytes,⁵³ elimination of prostate epithelial cells caused by androgen ablation⁵⁴ or activation of Fas receptor in Jurkat cells.⁵⁵ Administration of anti-apoptotic agents inhibited nucleolytic activities. Ca^{2+}/Mg^{2+} dependence, pH optimum above 7 and inhibition *in vitro* by Zn^{2+} were the characteristic features of nucleases that have been identified by these studies. These features lent credence to the idea that genome degradation and ensuing cell death are regulated by Ca^{2+} -dependent mechanisms. Nonetheless, cells may destroy their genome and die in the absence of Ca^{2+} signal. As we noted earlier, apoptotic agents can cause intracellular acidification and genome degradation without changing the intracellular Ca^{2+} concentration.^{2,14} Apoptosis of Jurkat cells caused by UV(C), exogenous ceramide or Fas receptor activation could be prevented by direct buffering of the acidic pH in the cytoplasm.¹ It had been thus inferred that cellular acidosis was providing favorable reaction conditions for the otherwise dormant low pH-dependent nuclease(s), which could perform the role of an apoptotic nuclease.^{26–30} This class of deoxyribonucleases, whose generic representative is DNase II, is characterized by (i) the dependence on acidic (<7) pH that is necessary for the nucleolytic activity, (ii) lack of stimulation by Ca and Mg ions and (iii) insensitivity to the inhibitory action of Zn ions *in vitro*. One would predict that this putative apoptotic nuclease should exhibit same activity in extracts isolated from control and apoptotic cells, since liabilities to intracellular pH should be observed only *in vivo*. As we have shown, this was not the case. The acidic nuclease activity was present in the nuclear fraction of untreated cells at a very low level while acidotic/apoptotic agents caused substantial activation of this enzyme. Whether the up-regulation of nuclease activity results from post-translational modifications or increased protein level remains to be established. Nuclease active forms, as revealed by zymography, are represented in HL-60 cells by four polypeptides of 56, 48, 45 and 40 kDa M_r. HeLa cells, on the other hand, possess 62 and 70 kDa forms of the acidic nuclease. Since the up-regulation of a low pH-dependent nuclease activity by apoptotic agents is not restricted to a particular cell line, it may therefore represent a more general event. Of note, anoxia also results in the up-regulation of a low pH-dependent nuclease in fibroblasts which is represented by DNase II-like 29–31 kDa polypeptides and a novel 44 kDa polypeptide.⁵⁶

Our enzyme is different from DNase II, as judged by its apparent molecular weight, although certainly belongs to the class of low pH-dependent nucleases. We have recently purified a 60 kDa acidic nuclease from human liver and skin fibroblasts that is clearly different from DNase II but similar to 56 kDa form found in HL-60 cells³⁹ (Famulski *et al*, manuscript in preparation). The diversity of active polypeptides representing this novel nuclease may reflect the cell type specificity and/or post-translational modifications.⁵⁷

The time course of nuclease activation in HL-60 cells shows good temporal correlation with the progression of apoptosis. Acidic nuclease activity usually reached its maximum within 4–8 h post treatment. At that time the population of subdiploid cells accounted for 30–70% of

total cell count. We therefore conclude that UV(C), etoposide or ceramide-induced genome degradation may be caused by low pH-dependent nuclease activity. Moreover, it has been extensively documented that cell death is preceded and then accompanied by the appearance of acidified cells. Exposure to acidotic/apoptotic agents results in 12–20% of cells succumbing to acidosis within 2 h. This number increases to 51–70% after 4 h.^{1,12,14,15,42} Hence, we suggest that the nuclease activation and the decrease in cellular pH occur simultaneously. However, nuclease up-regulation alone does not cause deleterious effects to the cell. Our findings demonstrate that anti-caspase/anti-acidotic agent Zn^{2+} rescues cells from genome degradation but does not alleviate the nuclease up-regulation. A similar conclusion can be reached when the effect of curcumin, an inhibitor of AP-1 transcription factor activity and apoptosis^{18,25,45} is evaluated. This drug rescues cells from being killed by ceramide and appears to offset rather than inhibit the ceramide-evoked acidic nuclease up-regulation. Clearly, the acidic nuclease which resides in HL-60 cells and constitutes the major nucleolytic activity therein³⁸ (this paper), is activated by acidotic/apoptotic agents *in vivo*. However, the mechanism down-regulating its activity remains to be established. It is conceivable that overexpression of anti-apoptotic proteins such as Bcl-2 or Ha-ras rather than Zn^{2+} or curcumin administration will alleviate the nuclease up-regulation caused by acidotic/apoptotic agents.^{13,52} Since activation of an acidic nuclease does not cause any deleterious effects when AP-1 transcription factor activity, caspase activation and intracellular acidification are down-regulated, we postulate that the acidic nuclease up-regulation alone is not a sufficient prerequisite for the execution of apoptosis.

Recently a novel mechanism responsible for the activation of a death nuclease has been discovered. The nuclease, CAD,⁵⁸ is present in living cells as an inactive complex with its inhibitor ICAD. Caspases activated by apoptotic stimuli cleave ICAD, thus allowing CAD to degrade chromosomal DNA.⁵⁹ Hence, apart from the postulated role of acidification and acidic nucleases in cellular death, a caspase-involving pathway that leads to genome degradation is being now considered. This pathway may be responsible for the progression of apoptosis occurring independently of cellular acidosis.

As alluded to before, the AP-1 transcription factor is associated with the initiation of apoptosis in HL-60 cells. We show here that the formation of its active complexes, induced by acidotic agents [UV(C), etoposide and ceramide], precedes apoptosis. Inhibition of AP-1, caused by the anti-caspase/anti-acidotic agent Zn^{2+} ^{14,42} or by curcumin, an inhibitor of AP-1 and NF κ B-dependent gene expression,⁴⁵ invariably led to the protection of cells from apoptosis. It has been recently documented that suppression of AP-1 activity by curcumin results from the inhibition of *c-jun* synthesis.²⁵ The role of *c-jun*, a component of dimers constituting AP-1 transcription factor, in the initiation of apoptosis is being now postulated. Increased *c-jun* expression apparently leads to the formation of AP-1 homodimers and induces apoptosis.^{20,25} It has been also demonstrated that *c-jun* acts upstream of the ICE/CED-3

family of cysteine proteases.²⁰ Zn^{2+} has been found to prevent the induction of active AP-1 complexes (this paper), activation of caspase-3⁴² while the caspase inhibitor alleviates acidification.^{12,15} Based upon these observations, we postulate that AP-1 induction, caspase activation and cellular acidification are in the same signaling pathway that is responsible for the initiation and progression of apoptosis. This pathway comprises Zn^{2+} -sensitive element(s) regulating AP-1 induction, caspase activation and ensuing acidification, while another pathway regulates the activity of a low pH-dependent nuclease and consists of Zn^{2+} -insensitive element(s).

Materials and Methods

Cell culture and treatment

HL-60 cells were maintained in Ham's F12 medium containing 10% fetal calf serum. Cells were grown to a density of 5×10^5 cells/ml and a total of 23×10^6 cells were washed and resuspended in 0.2 ml of phosphate-buffered saline (PBS), placed in a large culture dish and exposed to UV(C) light (254 nm) to a cumulative dose of 20 J/m². Total exposure time was 20 s. Immediately after irradiation, 40 ml of pre-warmed complete medium supplemented with 1 mM zinc sulfate was added to the cells. At various times post-irradiation (0–8 h) cells were collected for flow cytometric analysis of DNA content and isolation of nuclear proteins. Prior to etoposide treatment 23×10^6 cells were resuspended in 23 ml complete medium then incubated with the drug (20 μ g/ml, Sigma-Aldrich Canada Ltd., Oakville, ON) for 30 min, after which cells were washed with complete medium and resuspended in the same volume of ZnSO_4 -containing medium. At various times post-treatment (0–8 h) cells were collected for flow cytometric analysis of DNA content and isolation of nuclear proteins. For ceramide treatment, cells were incubated overnight in serum-free medium at a density 2×10^5 cells/ml. Ceramide (N-acetylsphingosine; Biomol Research Laboratories Inc, Plymouth Meeting, PA, USA) was added directly to the serum-free media at a final concentration of 10 μ M. Ceramide-treated cells were rescued from apoptosis by the addition of 50 μ M curcumin (Boehringer Mannheim, Laval, PQ), 30 min before cultures were treated with ceramide. At various times after treatment (0–8 h), cells were collected for flow cytometric analysis of DNA content and isolation of nuclear proteins.

Near confluent HeLa cells, grown in Ham's F12 medium supplemented with 10% fetal calf serum, were treated with 10 μ M ceramide in serum-free medium. In some cases cells were pretreated with 50 μ M curcumin prior to ceramide administration or coincubated with ceramide and 1 mM ZnSO_4 . UV(C) irradiation (20 J/m²) was achieved by exposing cells growing on culture dishes to UV light as described above. After 4 h of incubation cells were collected in lysis buffer as described below.

Nuclear extract preparation, electrophoretic mobility shift assay and zymography

Nuclear extracts were obtained as described previously.²³ Lysis buffer contained additionally 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μ g/ml of pepstatin, aprotinin and leupeptin. The extracts were stored at -70°C . Protein content was measured with the Bradford reagent (BioRad Laboratories, Richmond, CA, USA). Five μ g of nuclear proteins were assayed for the formation of active AP-1 complexes using a gel mobility-shift assay kit (Stratagene Cloning

Systems, La Jolla, CA, USA). AP-1 consensus oligonucleotide (Stratagene) was 5' labeled using polynucleotide kinase (Pharmacia Biotech Inc., Baie D'Urfe, PQ) and [γ - 32 P]ATP (NEN Research Laboratory Products, Boston MA, USA). Competition studies were performed by adding 50-fold molar excess of unlabeled oligonucleotide to reaction mixtures. Samples were analyzed by nondenaturing gel electrophoresis⁶⁰ and autoradiography. Autoradiographs that visualized EMSA assays were scanned by an IBM computer using a video camera. A digitized image analyzer (kindly provided by Dr. B. Palcic, BC Cancer Research Centre, Vancouver, BC) was then used to measure the intensity of the signals corresponding to AP-1 DNA-binding activity. Zymography assays were done according to³⁴ with some modifications. Ten per cent Laemmli SDS-acrylamide gels were co-polymerized with calf thymus DNA (0.2 mg/ml) and stored overnight at 4°C. Eighty μ g of nuclear or total cellular protein were mixed with an equal volume of the sample buffer and separated at 100 mV for 90 min. After washing and renaturation of proteins, gels were incubated in the activation buffer (50 mM sodium acetate, pH 5.2, 5 mM β -mercaptoethanol, 0.1% Triton X-100) overnight at room temperature. Acidic nuclease activity was detected by staining gels with ethidium bromide. Active polypeptides showed up as black bands on a fluorescent background. In order to determine the apparent molecular weight (M_r) of acidic nuclease prestained standards (New England BioLabs Ltd., Mississauga, ON) of known M_r values were used.

Total cell extract preparation and estimation of nucleolytic activity

Cells (4×10^6) were lysed in 500 μ l of 100 mM sodium acetate buffer pH 5.2 containing 1% NP-40, 5 mM 2-mercaptoethanol, 0.5 mM PMSF and 10 μ g/ml of pepstatin, aprotinin and leupeptin. After 15 min of incubation on ice, extracts were centrifuged at 4°C for 15 min at 14 000 r.p.m. in Eppendorf microcentrifuge. Supernatants were immediately frozen and used for zymography within a few days.

For estimation of nucleolytic activity 10 μ g of extracted nuclear proteins were incubated in the reaction mixture (100 μ l final volume) consisting of either 50 mM sodium acetate pH 5.2, 1 mM 2-mercaptoethanol and 33 μ g of calf thymus DNA, or 50 mM Tris-Cl pH 7.5, 50 mM NaCl, 3 mM CaCl_2 , 3 mM MgCl_2 , 1 mM 2-mercaptoethanol and 33 μ g of calf thymus DNA. Reactions (in duplicates) were carried out for 30 min and then terminated with the addition of 66 μ l of ice-cold 1.4 M perchloric acid. After a brief incubation on ice, samples were centrifuged at 4°C for 6 min at 14 000 r.p.m. in Eppendorf microcentrifuge. Absorbency at 260 nm was measured in 100 μ l aliquots with a Beckman diode-array spectrophotometer DU 7400. Measured values were corrected for non-specific absorbency by residual detergent present in nuclear extracts. An activity unit was defined as the amount of enzyme, present in 1 mg of protein extract, capable of release of 1 absorbency unit of acid-soluble oligonucleotides during 30 min.

Flow cytometric analysis of DNA content

Cells were harvested by centrifugation, washed once with PBS and fixed in 70% ethanol overnight at 4°C. Fixed cells were stained in PBS containing 50 μ g/ml of propidium iodide and 0.1 mg/ml of RNase (DNase-free; Pharmacia Biotech Inc., Baie D'Urfe, PQ) for 30 min. Analysis was performed within 30 min of staining. Fluorescence was detected with a FACSort cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with a doublet discrimination module. Propidium iodide fluorescence was excited with an argon ion laser emitting at 488 nm and collected by a photomultiplier tube after passing through a 585 nm

band pass filter. The same instrument settings were used for all experiments. Data were collected, using linear amplification for forward scatter, side scatter, and processed with CELLQuest Software (Becton-Dickinson, San Jose, CA, USA). Debris was excluded using forward and side scatter and a singlet gate was used in FL2-Width and FL2-Area dot plots.

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