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Characterization of Events Associated With Apoptosis/Anoikis Induced by Snake Venom Metalloproteinase BaP1 on Human Endothelial Cells

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Abstract Human endothelial EA.hy926 cells were incubated with BaP1, a hemorrhagic metalloproteinase purified from *Bothrops asper* snake venom. Since the first hour of incubation with the proteinase, cells started showing DNA fragmentation, detected by a terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling (TUNEL)-based photometric enzyme-linked immunosorbent assay (ELISA). At later times, DNA fragments were predominantly located outside the cells, evidencing plasma membrane rupture. DNA fragmentation was completely abolished by Batimastat, a potent inhibitor of metalloproteinase enzymatic activity. Apoptosis induced by BaP1 on endothelial cells was independent of two Bcl-2 family members (anti-apototic Bcl-xL and pro-apoptotic Bax), that did not show any changes in their expression during a 24 h-treatment period. Interestingly, IκBα, an inhibitor of NFκB, decreased after 24 h of treatment, suggesting further activation of the transcription factor. When some elements of the apoptotic extrinsic pathway were assessed, it was observed that procaspase-8 completely disappeared after 24 h of treatment with BaP1, probably indicating its activation by a death receptor, whereas caspase-8 inhibitor, cellular FLICE-inhibitory protein ($cFLIP_L$), increased its expression since the first hours of BaP1 incubation. In conclusion, treatment of human endothelial cells with BaP1 induces apoptosis/anoikis, independently of Bcl-2 family members Bax and Bcl-xL and associated with caspase-8 activation and $cFLIP_L$ up-regulation. Apoptosis was completely dependent on BaP1 enzymatic activity. Similarities between this and other endothelial cell anoikis-related systems suggest that BaP1 and other snake venom metalloproteinases may be useful experimental tools in the study of death-related events that occur when adherent cells loose contact with extracellular matrix. J. Cell. Biochem. 94: 520–528, 2005.

Key words: metalloproteinase; snake venom; anoikis; apoptosis; endothelial cells

Anoikis is a poorly characterized form of apoptosis that occurs when adherent cells lose their integrin-mediated attachment to the extracellular matrix (EM) [Meredith et al., 1993; Grossman, 2002]. Anoikis has been associated with the activation of the extrinsic apoptotic pathway, which in endothelial cells is likely to occur by a Fas-mediated mechanism [Aoudjit and Vuori, 2001]. Endothelial cell detachment from the matrix results in an increase of cell

Abbreviations used: TUNEL, terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling; ELISA, enzyme-linked immunosorbent assay; IκB, NFκB inhibitor; NFκB, nuclear factor κB; cFLIP, cellular FLICE-inhibitory protein; EM, extracellular matrix; poly-HEMA, poly(2-hydroxyethyl methacrylate); MMP, matrix metalloproteinase; ADAMs, protein with a disintegrin and a metalloprotease domain; TNFα, tumor necrosis factor alpha; TACE, TNFα-converting enzyme; SVMPs, snake venom metalloproteinases; MTT, 3-(4,5-dimethyl-2-thiazolyl(-2,5-diphenyl-2H-tetrazolium bromide); PBS, phosphate-buffered saline; EDTA, ethylenediamine-tetraacetic acid; BrdU, 5′-bromo-2′-deoxy-uridine; SDS–PAGE, sodium duodecyl sulfate–polyacrylamide gel electrophoresis; HUVECs, human umbilical vein endothelial cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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surface Fas expression and down-regulation of cellular FLICE-inhibitory protein (cFLIP), an endogenous caspase-8 inhibitor, further supporting the concept that the extrinsic apoptotic pathway is involved in this event.

Two model systems have been commonly used in the study of detachment induced apoptosis: (a) treatment with proteinases, such as trypsin, for various time intervals, and (b) culturing adherent cells on hydrogels such as poly(2-hydroxyethyl methacrylate), known as poly-HEMA. In the first case, treatment with proteinases leads to a complete detachment from EM, together with degradation of proteins from plasma membranes and EM. This system has the problem that proteinases such as trypsin are strongly inhibited by serum components. Therefore, culture conditions have to be adjusted in order to reduce this effect, with the consequence that long-term incubation in serum-deprived conditions favors apoptosis associated with the withdrawal of growth factor-dependent survival signals. The second model consists in avoiding cell attachment after trypsin treatment by covering the plates with hydrogels, substances that do not allow cell adherence. These are highly artificial conditions for the cells in terms of interactions with EM, and are likely to induce unknown effects due to the charge of these substrata. Culturing fibroblasts in poly-HEMA, for example, decreases mRNA synthesis and collagen production and causes complete inhibition of cell proliferation [Peluso et al., 1997]. Hence, there is a need to develop new experimental models of anoikis in order to further explore the mechanisms behind this type of cellular death.

Zinc-dependent metalloproteinases comprise a wide group of enzymes present in many biological fluids [Maskos and Bode, 2003]. Some of them, such as matrix metalloproteinases (MMPs), cleave various EM proteins and exert a number of relevant physiological roles by releasing peptides from endogenous proteins and by revealing cryptic sites [Blobel, 2000]. Another group of metalloproteinases, the proteins with a disintegrin and a metalloprotease domain (ADAMs), exert diverse roles in cell–cell interactions and in shedding of membrane proteins, thereby releasing biologically active peptides [White, 2003]. The enzyme ADAM-17, also known as tumor necrosis factor alpha (TNFα)-converting enzyme (TACE), releases active TNFα from its membrane precursor [White, 2003]. Snake venom metalloproteinases (SVMPs), on the other hand, are able to cleave soluble TNFα in vitro [Moura da Silva et al., 1996; Clissa et al., 2001]. ADAMs and SVMPs comprise the group of 'reprolysins,' which share a similar multi-domain organization characterized by the presence of a metalloproteinase domain followed by disintegrin-like and cysteine-rich domains and, in the case of ADAMs, by additional domains including a transmembrane region [Stone et al., 1999]. Some SVMPs contain metalloproteinase, disintegrin-like and cysteine-rich domains, whereas others only display the metalloproteinase domain [Bjarnason and Fox, 1994]. Taking into account the similarities between some endogenous metalloproteinases and SVMPs, the latter may become useful tools in the study of metalloproteinase-induced cell detachment leading to apoptosis.

Previous communications have demonstrated that apoptosis is induced in endothelial cells upon incubation with SVMPs [Masuda et al., 2000, 2001; Wu et al., 2001; Araki et al., 2002; Wu and Huang, 2003; You et al., 2003]. In some of these cases, apoptosis did not occur when proteolytic activity was inhibited [Araki et al., 2002], whereas in other cases such as with a multi-domain SVMP from the venom of *Gloydius halys*, inhibition of catalysis did not completely abrogate the apoptotic effect [You et al., 2003]. However, the mechanism of SVMP-induced apoptosis remains poorly characterized. BaP1 is a SVMP, composed by the metalloproteinase domain only, present in the venom of the pit viper *Bothrops asper*, the most important snake from the medical standpoint in Central America and southern México [Watanabe et al., 2003]. BaP1 is highly abundant in this venom [Gutiérrez et al., 1995] and induces a number of relevant pathological effects, including hemorrhage, myonecrosis, edema, blistering, dermonecrosis, and inflammation [Rucavado et al., 1995, 1998, 2002; Farsky et al., 2000].

The aim of this work is to characterize apoptotic events associated with the effect of SVMP BaP1 on endothelial cells in terms of the type of cell death pathway triggered and some of the molecules involved in the process, and to correlate these findings with other reported events that occur during endothelial cell-induced anoikis, in the search for new tools to study the interactions between cells and EM.

MATERIALS AND METHODS

Reagents and Antibodies

Dulbecco essential medium, fetal bovine serum and 3-(4,5-dimethyl-2-thiazolyl(-2,5-diphenyl-2H-tetrazolium bromide) (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). Antibodies against Bcl-xL (H5, sc-8392), Bax (B9, sc-7480), and IκBα (C-21, sc-371) were obtained from Santa Cruz (San Diego, CA). Antibodies against caspase-8 (AB1879), and cFLIP (AB16963) were obtained from Chemicon International (Temecula, CA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit whole IgG was purchased from Jackson Immuno-Research Laboratories, Inc. (West Grove, PA). Cellular DNA Fragmentation enzyme-linked immunosorbent assay (ELISA) (Cat. No. 1585045) was obtained from Roche Molecular Biochemicals (Mannheim, Germany). BaP1 was purified according to Gutiérrez et al. [1995]. British Biotech Pharmaceuticals Ltd. kindly provided Batimastat.

Cell Culture and Treatment

Human vascular endothelial cells EA.hy926 (donated by Dr. Jorg Hacker, University of Wurzburg, Germany) were maintained in Dulbecco essential medium supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 IU/ml penicillin, and amphotericin B in a 37°C humidified incubator under an atmosphere of 7% CO_2 in air. For the experiments, cells were plated on 96-well plates to confluence and were allowed to adhere overnight. Various concentrations of BaP1 were added to the plates containing confluent cell monolayers in 100 μl of fresh medium and were incubated for different time periods as indicated in the figures. Before using, Batimastat was dispersed by sonication in phosphate-buffered saline (PBS) solution, pH 7.2 containing 0.01% Tween-80 detergent at a final concentration of 2 mM. Inhibition experiments were done with 160 μM Batimastat.

Assessment of Cytotoxicity by MTT Assay

Cells were treated with increasing concentrations of BaP1 during three time periods (1, 4, and 5 days). Then, 10 μl of MTT reagent (5 mg/ml in PBS) was added to the culture medium and, after 2 h at 37°C, medium was carefully removed (without collecting any of the detaching cells) and 100 μl of 95% ethanol was added to the wells to dissolve the formazan crystals. Absorbances were read at 570 nm and results were expressed as viability percentages, using samples incubated with PBS as 100% viability value.

Assessment of Apoptosis by the Photometric ELISA for Detection of BrdU-Labeled DNA Fragments

Briefly, cells were removed from the plates with trypsin–ethylenediamine-tetraacetic acid (EDTA) solution and labeled for 4–6 h with 5′-bromo-2′-deoxy-uridine (BrdU) at 37°C. After removing the medium containing BrdU, cells were plated and allowed to adhere overnight. After addition of 200 μg/ml of BaP1 for different time intervals, supernatants were carefully collected (without removing the cells) and kept at −70°C for no more than 3 days. Cells were lysed with the appropriate solution for 30 min and the extracts were removed and kept also at −70°C. The ELISA was performed according to the manufacturer's instructions and the results are expressed as absorbance at 450 nm.

Western Blot Analysis

After treatment with BaP1 (200 μg/ml), endothelial cells were removed from the plates, resuspended in buffer (50 mmol/L Tris-HCL, 150 mmol/L NaCl, pH 8.0) and sonicated for 1–2 min on ice. After measuring protein concentration, equal amounts of protein from extracts were separated using 11 or 15% polyacrylamide gels (depending on the molecular mass of the protein to be detected) by sodium duodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose membranes. Blots were blocked with 2% dried milk and incubated for 2 h with the primary antibodies at concentrations of 1:100 or 1:200. After washings, blots were incubated with peroxidase-conjugated anti-mouse and anti-rabbit IgG for two additional hours at concentrations of 1:2,000 (anti-mouse) and 1:3,000 (anti-rabbit). Blots were developed adding 2 mg/ml 4-chloronaphtol in 0.02 M Tris, 0.5 M NaCl, pH 7.5 (containing 0.025% H_2O_2).

Statistical Analysis

The Student's *t*-test was performed to determine the significance of the differences between means of two experimental groups in the DNA fragmentation tests (time–response curve) using Systat 9.0 Statistics Program. When more than two groups were compared a two-way

ANOVA was applied, followed by Tukey–Kramer comparison of pairs of means.

RESULTS

Cytotoxicity Induced by BaP1 on Endothelial Cells

According to the MTT assay, cells were highly resistant to BaP1, since during a 24 h period of incubation with various proteinase concentrations, no significant toxicity was detected (Fig. 1). After 4 and 5 days of treatment, a dose-dependent cell death was observed, even at the lowest BaP1 concentrations tested. Since 100 μg/ml of BaP1 was not enough to induce cytotoxicity at 24 h (according to MTT results), we decided to carry out the rest of the experiments with a higher concentration (200 μg/ml).

DNA Fragmentation Induced by BaP1 on Endothelial Cells

Results of DNA fragmentation assay for endothelial cells treated with 200 μg/ml BaP1 showed that, up to 6 h, apoptosis was the main mechanism of cell death observed, since DNA fragments were present only inside the cells, showing that plasma membranes were still intact (Fig. 2). At 24 h (see below), DNA fragments were observed mainly in the supernatant, indicating secondary necrosis. The treatment induced an evident cell detachment that was occurring simultaneously with the onset of apoptosis.

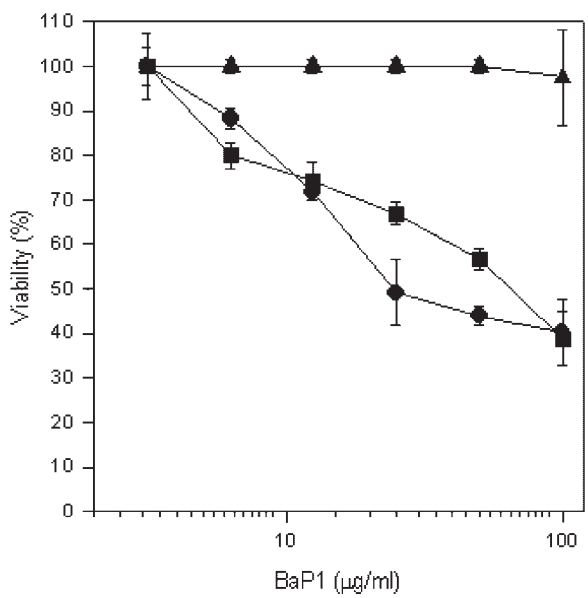

Fig. 1. Cytotoxicity induced by BaP1 on human endothelial cells. Cells were incubated with increasing concentrations of metalloproteinase BaP1 for (▲) 1, (■) 4, and (●) 5 days. After incubation for 2 h with MTT, culture medium was carefully removed, without collecting any detached cells and formazan crystals were dissolved in 95% ethanol. Plates were read at 570 nm and viability percentages were calculated using cells incubated with phosphate-buffered saline (PBS) as 100% viability value. Results are expressed as mean ± SE of triplicates of one representative experiment.

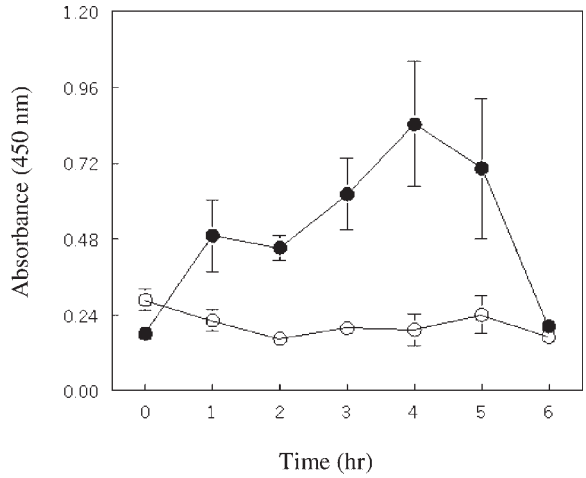

Fig. 2. Apoptosis of endothelial cells induced by treatment with BaP1. Cells were treated for different time intervals with 200 μg/ml of BaP1, and DNA fragments were detected by ELISA on the supernatant (○) and cell extracts (●). The presence of DNA fragments exclusively in cell lysates implies cells are dying by apoptosis. Results are expressed as mean ± SE of triplicates of one representative experiment.

Effect of BaP1 Treatment on the Expression of Apoptosis-Related Genes: *Bcl-xL* and *Bax*

As shown in Figure 3, treatment with BaP1 (200 μg/ml) during 24 h did not induce detectable changes in the expression of anti-apoptotic protein Bcl-xL and pro-apoptotic protein Bax. The same was observed at 48 h of treatment (results not shown).

Effect of BaP1 Treatment on IκB Processing

To determine the possibility of IκB being cleaved, we measured the decrease in this protein levels. Figure 4 shows that IκBα was down-regulated in these cells after 24 h of treatment, probably indicating degradation by proteinases, although this effect was not seen at earlier times (6 h).

Effect of BaP1 Treatment on the Activation of Caspase-8 and cFLIP Expression

Figure 4 clearly shows that caspase-8 proform concentration decreased after 24 h of BaP1

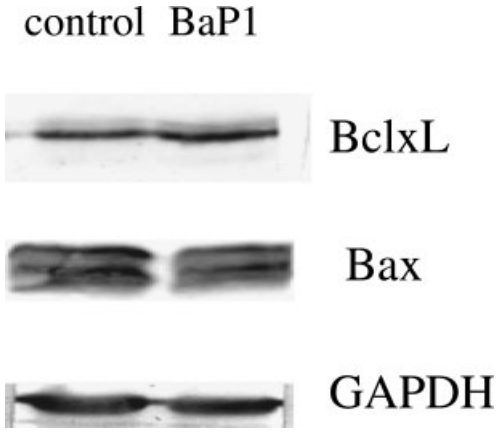

Fig. 3. Expression of Bcl-xL and Bax on endothelial cells after 24 h of treatment with BaP1. Cells were treated with 200 μg/ml of BaP1 and extracts were run on sodium duodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose. Equal loading was assured by measuring protein concentration of the samples and using an antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Control corresponds to cells incubated with PBS instead of BaP1. Cell extracts were tested with antibodies to Bcl-xL and Bax. Results show one of three representative experiments.

treatment, which could indicate that this caspase is being activated. At 6 h, however, the levels of procaspase-8 were still normal. Expression of endogenous caspase-8 inhibitor, the long form of cFLIP, increased at 6 h of treatment, an effect that became more evident at 24 h (Fig. 4).

Effect of Metalloproteinase Inhibitor Batimastat on DNA Fragmentation Induced by BaP1

When DNA fragmentation induced by BaP1 was tested in the presence of the metalloproteinase inhibitor Batimastat, there was a complete inhibition of the effect induced by the proteinase (Fig. 5). The inhibition was statistically significant ($P < 0.01$). Moreover, no cell detachment occurred in the presence of Batimastat. At the time points recorded (24 and 48 h) DNA fragments were observed only in the supernatants of samples treated with BaP1 alone, indicating that cells were already suffering membrane damage characteristic of necrosis, after having undergone apoptosis previously (Fig. 2).

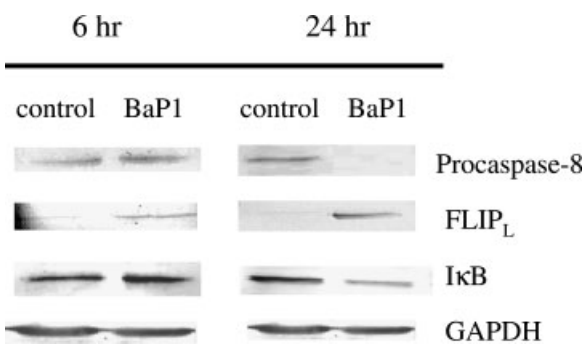

Fig. 4. Expression of procaspase-8, FLIP$_L$ and IκB on endothelial cells after 6 and 24 h of treatment with BaP1. Cells were treated with 200 μg/ml of BaP1 and extracts were run on SDS–PAGE and transferred to nitrocellulose. Equal loading was assured by measuring protein concentration of the samples and using an antibody to GAPDH. Control corresponds to cells incubated with PBS instead of BaP1. Cell extracts were tested with antibodies to procaspase-8, FLIP$_L$ and IκB. Results show one of three representative experiments.

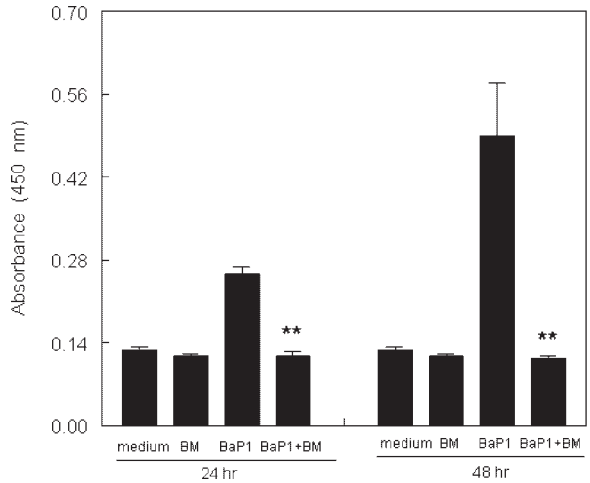

Fig. 5. Effect of metalloproteinase inhibitor Batimastat (BM) on DNA fragmentation induced by BaP1 in endothelial cells. Cells were treated for 24 and 48 h with BaP1 previously incubated for 15 min with Batimastat. DNA fragmentation was determined as described in Figure 2. Bars represent DNA fragments detected in cell supernatants. Results are presented as mean ± SE of triplicates of one representative experiment. $^{**}P < 0.01$ by ANOVA. There were no significant differences in DNA fragmentation between cells incubated with PBS, Batimastat alone or with BaP1 + Batimastat.

DISCUSSION

Snake venom metalloproteinase BaP1 induces apoptosis/anoikis in endothelial cells within the first hours of incubation, and this process leads to plasma membrane rupture, an event associated with secondary necrosis. Our findings agree with previous communications reporting the ability of SVMPs to induce apoptosis in endothelial cells [Masuda et al., 2000, 2001; Wu et al., 2001; Wang et al., 2003; You et al., 2003], and with the observation that SVMPs initially induce cell detachment from the substratum, whereas plasma membrane damage occurs only at later time intervals [Borkow et al., 1995; Marsh et al., 1995].

We also observed that there is no correlation between MTT cytotoxicity assay and DNA fragmentation ELISA, since according to the former technique BaP1 is not cytotoxic at concentrations as high as 100 μg/ml after 24 h of treatment, whereas DNA fragmentation was observed since the first hour of incubation with the proteinase. Lack of toxicity according to MTT results was the reason for using a relatively high concentration of BaP1 (200 μg/ml) for testing apoptosis and changes in the expression of several apoptotic-related genes. MTT assay results agree with previous reports in the literature showing that SVMPs are not toxic for endothelial cells in time periods of 24 h or less [Lomonte et al., 1994; Borkow et al., 1995; Rucavado et al., 1995]. It is important to point out that all these studies used membrane rupture-based techniques to measure cytotoxicity, such as Trypan blue exclusion or lactate dehydrogenase release. As we observed in this study, apoptosis occurs early (since the first hour of treatment with BaP1), whereas membrane disruption is only evident at 24 h or later. MTT assay does not discriminate between apoptosis and necrosis, since it determines oxidative activity inside the cells, not only associated with mitochondrial redox power, but also with the activity of oxidative enzymes present in the plasma membrane [Bernas and Dobrucki, 2000]. Also, MTT assay is not very sensitive, which further explains the apparent discrepancy of the results between this assay and the DNA fragmentation ELISA.

Anchorage to the EM has been implicated in cell survival since disruption of this contact induces apoptosis/anoikis in endothelial and other epithelial cells [Meredith et al., 1993; Frisch and Francis, 1994; Werb, 1997]. Inhibition of apoptosis by oncogenic transformation has been suggested as a mechanism for achieving anchorage-independent growth potential in adherent cells that have lost substrate attachment [Ruoslahti and Reed, 1994]. Until recently, the molecular mechanisms responsible for regulating endothelial cell anoikis were unknown; however, Aoudjit and Vuori [2001] demonstrated that anoikis in human umbilical vein endothelial cells (HUVECs) was associated with Fas/FasL system involving caspase-8 activation. They showed that cFLIP, an endogenous inhibitor of caspase-8, was also involved. Previously, other investigators [Frisch, 1999; Rytomaa et al., 1999] had demonstrated that anoikis in epithelial cells was blocked by inhibitors of caspase-8.

In our experiments, we clearly showed that procaspase-8 levels decreased in endothelial cells treated with BaP1 after 24 h of treatment. Interestingly, we did not observe any changes in the expression of procaspase-8 after 6 h of BaP1 treatment, even when cells were already dying by apoptotic mechanisms at this time interval. We postulate that the decrease in procaspase-8 is related with its activation, even though an alternative explanation could be simply that protein synthesis decreases. We also observed that caspase-8 inhibitor $cFLIP_L$, which is expressed at low levels in this endothelial cell line, increases its expression early in the treatment, and is significantly up-regulated after 24 h of incubation with BaP1. Interestingly, recent reports have suggested that $cFLIP_L$ could display an apoptotic function when is overexpressed, by promoting caspase-8 activation [Chang et al., 2002; Micheau et al., 2002; Boatright et al., 2004]. Clear up-regulation of $cFLIP_L$ observed in endothelial cells incubated with BaP1 suggests that this protein may induce apoptosis. It is suggested that $cFLIP_S$ rather than $cFLIP_L$ confers resistence to some apoptotic stimuli, whereas $cFLIP_L$ can function to enhance caspase-8 activation [Boatright et al., 2004].

It is likely that BaP1 induces apoptosis/anoikis through the extrinsic pathway mediated by a death receptor that, according to several studies, could be Fas, or alternatively, TNFα receptor. It has been previously shown that SVMPs cleave and activate TNFα in vitro [Moura da Silva et al., 1996; Clissa et al., 2001]. Even though we have not been able to show the same effect in vivo [Rucavado et al., 2002], this possibility cannot be completely ruled out. In agreement with our findings, incubation of endothelial cells with sublytic concentrations of two metalloproteinase-containing snake venoms induces up-regulation of genes involved in the Fas ligand/TNFαR apoptotic pathway [Gallagher et al., 2003]. Alternatively, we cannot rule out the possibility that the effect could be triggered, not by death receptors, but instead by the recruitment of caspase-8 directly through unligated integrins [Stupack et al., 2001]

We also tested whether IκB was being degraded as evidence of NFkB activation. Even though NFκB activation was not measured

directly, IκB expression has been used as an indirect method [Kim et al., 2002]. It was evident that IκB levels decrease after 24 h treatment with BaP1, which could be indicating that a survival signal mediated by NFκB is being triggered. This signal, however, does not seem to be enough to block apoptosis mediated by EM contact loss. IκB, on the other hand, has been shown to be a substrate for caspases [Wang and Passaniti, 1999], an event that seems to be independent of the proteasome degradation that leads to NFκB activation. Since we cannot rule out the possibility that IκB down-regulation is the result of changes in its synthesis, it cannot be concluded with certainty whether this event actually represents a survival signal mediated by NFκB in these cells.

Members of Bcl-2 family of proteins, such as Bcl-xL, have been found to protect epithelial cells against anoikis [Rytomaa et al., 1999; Rosen et al., 2000]. However, in some cell lines such as LnCap, anoikis was completely independent of Bcl-2 family members [Bondar and McConkey, 2002]. We observed that BaP1-induced apoptosis/anoikis was independent of Bax and Bcl-xL expression, since no changes were detected in the levels of both proteins, even at 48 h of treatment, several hours after DNA fragmentation was detected.

SVMPs comprise various classes, classified according to their domain composition [Bjarnason and Fox, 1994]. P-I metalloproteinases are made of the metalloproteinase domain only, whereas P-II and P-III groups include enzymes which have, in addition to the metalloproteinase domain, a disintegrin/like domain (class II) or disintegrin-like and cysteine-rich domains (class P-III). Such additional domains interact with integrins and mediate various effects such as inhibition of platelet aggregation or cell adhesion [Kamiguti et al., 1996; Moura da Silva et al., 2001]. Interestingly, both P-I and P-III snake venom metalloproteinases are able to induce apoptosis of endothelial cells. In the case of P-I enzymes, inhibition of catalytic activity abrogates their ability to induce apoptosis [Wu et al., 2001]. In contrast, halysase, a P-III enzyme from the venom of *G. halys*, induces apoptosis even when catalytic activity is absent [You et al., 2003]. It is, therefore, likely that disintegrin-like or cysteine-rich domains of P-III SVMPs promote endothelial cell apoptosis, probably by interfering with cell–EM interaction mediated by integrins. The proapoptotic activity of BaP1 was inhibited by incubation with the metalloproteinase inhibitor Batimastat, in agreement with the fact that it is a P-I metalloproteinase containing only the catalytic domain [Watanabe et al., 2003].

In the case of P-III metalloproteinase halysase, it has been shown that the apoptotic mechanisms on endothelial cells include not only the activation of caspase-3, but also alterations in Bcl-xL and Bax protein levels [You et al., 2003]. Contrary to what we observed with BaP1, You et al. [2003] were able to show both Bcl-xL down-regulation and Bax up-regulation after 6 h of treatment. These differences could be explained based on the fact that BaP1 lacks disintegrin-like and cysteine-rich domains, which seem to be important for the induction of apoptosis by halysase. It is likely that, since disintegrin-like and metalloproteinase domains are important for inducing apoptosis, and since both are able to induce the effect, the cytotoxic mechanisms operating in this case may be different from the ones induced by a metalloproteinase containing only one of these domains. In addition, it has been proposed that the disintegrin-like domain could modulate the catalytic activity of the enzyme by regulating the hydrolysis of EM proteins, determining its substrate specificity [Jeon and Kim, 1999], thus suggesting that the effects of SVMPs in cells may involve the interplay of various domains present in these enzymes.

SVMPs are responsible for the hemorrhagic activity that characterizes envenomations by snakes of the family Viperidae [Bjarnason and Fox, 1994; Gutiérrez and Rucavado, 2000]. In experimental animals such effect occurs within few minutes after injection [Ownby et al., 1978; Moreira et al., 1994], probably before apoptotic events develop. Thus, the role of the apoptosis/anoikis phenomenon described in this work in snakebite envenomation associated endothelial cell pathology is not clear at present. It might be that, despite occurring after the onset of microvessel damage leading to hemorrhage in tissues affected by metalloproteinases, apoptosis may play a role in endothelial pathology on a broader context, affecting tissue perfusion and processes involving inflammation, tissue repair, angiogenesis, and regeneration. The role of endothelial cell apoptosis in the pathogenesis of venom-induced tissue damage remains to be investigated.

In conclusion, snake venom metalloproteinases such as BaP1 may become useful experimental tools to investigate the cellular mechanisms associated with apoptosis/anoikis. It is suggested that BaP1 induces endothelial cell apoptosis/anoikis through the extrinsic pathway, probably mediated by death receptors. The role of endothelial cell apoptosis in the tissue damage associated with snakebite envenomation deserves further investigation.

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