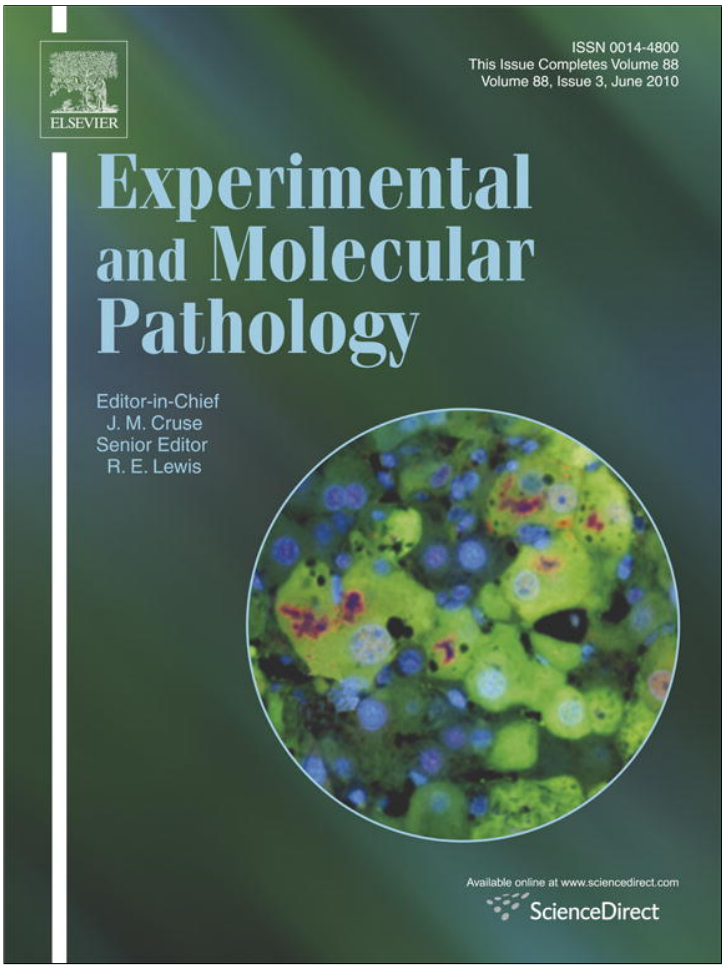

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Cell death induced by *Bothrops asper* snake venom metalloproteinase on endothelial and other cell lines

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ABSTRACT

Two adherent cell lines, BAEC and HeLa, and non-adherent Jurkat, were treated with snake venom metalloproteinase BaP1 to determine whether cytotoxicity, previously reported for this toxin, could be mediated by the process of anoikis. It was observed that there was no correlation between the ability of this toxin to induce loss of adherence, and the cytotoxic effect, since concentrations that do not induce loss of adherence (3–6 μg/mL), were able to trigger 50% of cytotoxicity in BAEC. In the case of HeLa, where toxicity was very low (less than 20% at maximun concentrations and times of exposure), significant detachment and no toxicity was observed at concentrations of 1.5 μg/mL, showing also no correlation between both events.

We also observed differences between BAEC toxicity measured by XTT reduction and DNA fragmentation determined by flow cytometry (as an indicator of apoptosis), since concentrations that induce 100% of cytotoxicity barely showed any DNA fragmentation (12% at 24 h), suggesting that if apoptosis was involved, DNA damage is still not present, although chromatin condensation, another indicator of apoptosis, is observed in 40% of the cells. Inhibition of BAEC cytotoxicity by caspase inhibitors indicate that apoptosis is playing a role in this process, but other mechanisms of cell death could be participating also.

Another way to determine whether the mechanism of cell death was related to anoikis was using a non-adherent cell line, which should show substrate independence. We determined by TUNEL that at 50 μg/ml BaP1 triggered 50% of apoptosis at 96 h, an effect that was seen earlier, suggesting also that if this toxin was inducing apoptosis in a non-adherent cell line, the mechanism could not be related to loss of attachment. Cell cycle arrest in S phase was also observed in Jurkat cells, an effect that could be leading to apoptosis.

In conclusion, since there was no correlation between cell detachment and cytotoxicity (and apoptosis) in adherent cell lines and due to the ability of BaP1 to induce apoptosis in a non-adherent cell line, we suggest that this enzyme is toxic by a mechanism not related to anoikis, and that in the case of Jurkat cells, it is likely to be related to its ability to induce cell cycle arrest. Processes other than apoptosis could be also involved in the cell death mechanism mediated by BaP1 on BAEC.

Introduction

Zinc-dependent metalloproteinases consist of a wide group of enzymes present in many biological fluids (Maskos and Bode, 2003). Some of them, such as matrix metalloproteinases (MMPs), cleave several proteins, including cellular receptors, extracellular matrix (ECM) and plasma membrane proteins, which have a number of relevant physiological roles (Blobel, 2000; Mannello et al., 2005; Kirkin et al., 2007). Another group of metalloproteinases, the ADAMs, play diverse roles in cell–cell interactions and in shedding of membrane proteins, thereby releasing biologically active peptides (White, 2003). MMPs, ADAMs and snake venom metalloproteinases (SVMPs) comprise the superfamily of metzincins, and the last two groups comprise the group of 'reprolysins', which share a similar multi-domain organization, which is 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; Fox and Serrano, 2005). Some SVMPs have metalloproteinase, disintegrin-like and cysteine-rich domains, whereas others only display the metalloproteinase domain (Bjarnason and Fox, 1994; Fox and Serrano, 2005). Taking into account the similarities between metalloproteinases activities, SVMPs may become useful tools in the study of proteinase-induced cell detachment and the processes that follow it.

Previous publications have demonstrated that SVMPs can induce apoptosis in endothelial cells (Masuda et al., 2000; Wu et al., 2001;

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Araki et al., 2002; Wu and Huang, 2003; You et al., 2003; Díaz et al., 2005; Tanjoni et al., 2005; Trummal et al., 2005). In some of these cases, apoptosis did not occur when proteolytic activity was inhibited (Araki et al., 2002; Díaz et al., 2005; Tanjoni et al., 2005), 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).

The mechanism of SVMP-induced apoptosis remains poorly characterized. Anchorage to the ECM has been associated with cell survival and disruption of this contact induces apoptosis/anoikis in endothelial and 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). In many of the reports SVMPs-induced cell death has been indirectly associated to the anoikis process but other studies suggest that cell death could be detachment-independent (Araki et al., 2002; Wu and Huang, 2003; Wan et al., 2006).

BaP1 is a SVMP that contains only the metalloproteinase domain. This toxin is present in the venom of the pit viper *Bothrops asper*, the most important snake, from the medical point of view, in Central America and southern México [Watanabe et al., 2003]. BaP1 is highly abundant in the 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).

We had previously shown that snake venom metalloproteinase BaP1 induces apoptosis in Ea.hy.926 endothelial cells within the first hours of incubation, and that this process led to plasma membrane rupture, an event associated with secondary necrosis. We observed that the apoptotic effect was associated with caspase-8 activation and was also independent of Bcl-xL and Bax (Díaz et al., 2005).

Since the association between SVMP-induced apoptosis and cell substrate detachment has been made in most part, indirectly, we decided to evaluate the effects induced by BaP1 in cell lines that are adherent and other that remain in suspension, trying to elucidate whether the mechanism of action of this proteinase could be due to anoikis or to another mechanisms unrelated to the survival signals associated to substrate interactions.

Materials and methods

Reagents and BaP1

Dulbecco essential medium (DMEM), RPMI-1640, fetal bovine serum, propidium iodide (PI), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), 2,3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) monodansylcadaverine (MDC) and Hoechst 33258 were obtained from Sigma Chemical Co. (St. Louis, MO). Calcein-AM was obtained from Invitrogen (Barcelona, España). For TUNEL assay, fluoresceine-12-deoxy-2-uridine triphosphate (FITC-dUTP) and terminal deoxynucleotidyl-transferase (TdT) were obtained from Roche Molecular Biochemicals (Mannheim, Germany). BaP1 was purified from *Bothrops asper* snake venom according to Gutiérrez et al. (1995).

Cell culture and treatment

Bovine aortic endothelial cells (BAEC), cervix adenocarcinoma cells (HeLa) and T cell leukemia Jurkat cells (obtained from ATCC, Rockville, MD) were maintained in DMEM or RPMI-1640 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine and 100 IU/ml penicillin/streptomycin, in a 37 °C humidified incubator under an atmosphere of 7% CO_2 in air. Various concentrations of BaP1 were added to the plates containing 80% confluent cell monolayers in fresh medium and incubated for different time periods as indicated in the figures.

MTT and XTT reduction assays

To determine viability, cells, cultured in 96 well plates were treated with different concentrations of BaP1 and a solution containing XTT and phenazine methosulfate (final concentration of 0.2 mg/mL and 0.2 M, respectively) was added to the wells. After 3 h of incubation at 37 °C in the dark, absorbance was read at 450 nm and a blank well containing only medium and the labeling mixture was included in the readings to background correction.

To determine BAEC loss of adherence, cells were culture in 96 well plates and after treatment with BaP1, they were washed four times with PBS and MTT was added to each well at a final concentration of 0.45 mg/mL for 1 h at 37 °C. Medium was removed and 95% ethanol was added to the wells with the purpose of dissolving formazan crystals. Absorbances were read at 570 nm and results were expressed as viability percentage.

Calcein uptake assay

To determine HeLa loss of adherence, cells were cultured in 96 well plates and after treatment with BaP1, they were washed twice with PBS. Calcein-AM was added to the cells to a final concentration of 1 μM for 30 min at 37 °C. Cells were washed again two times with PBS and fluorescence was analyzed in 4 points of the well using a Tecan Pro-Fluorometer (535 nm).

Studies with inhibitors

BAEC were cultured on 96-well plates and incubated with 100 μL of different concentrations of BaP1 for 24 h, in the presence or absence of NH_4Cl (10 mM). After incubation, 25 μL of the labeling mixture (1 mg/mL XTT chromophore and 1 mM phenazine methosulfate in PBS, pH 7.2), were added to each well. After approximately 3 h of incubation at 37 °C in the dark, the absorbance was recorded at 450 nm. The results are expressed as viability percentages using cells treated with PBS as negative controls (100% of viability).

In the case of the experiments with protease inhibitors, BAEC were cultured in 96-well plates and pre-incubated for 2 h with a pan-caspase inhibitor (Z-VAD-FMK), a caspase-8 inhibitor (Z-IETD-FMK), a caspase-3 inhibitor (Z-DEVD-FMK) and an inhibitor of calpains (ALLN), at a final concentration of 30 μM. Subsequently, medium was removed and cells were treated with 200 μg/mL BaP1 for 24 h. After treatment, cell viability was evaluated by XTT reduction (as indicated above).

Cell cycle analysis

After treatment with BaP1, cells collected, washed with PBS and fixed with ethanol (70%) for 24 h at 4 °C. Cells were treated with RNase-A (50 U/ml) and labeled with PI (20 μg/ml) for 3 h at room temperature. The percentage of cells in each phase of the cell cycle was determined by flow cytometry in an EPIC XL Analyzer (Beckman-Coulter) or a FACScallibur (Beckton-Dickinson).

Fluorescence labeling to determine chromatin condensation, presence of vacuoles and membrane rupture

Cells were cultured on 24-well plates and incubated with 100 μL of different concentrations of BaP1 for 24 h. After treatment, medium was carefully removed, cells were washed with PBS and labeled for 20 min, at 37 °C in the dark, with a fluorochrome mixture containing monodansylcadaverine (0.05 mM), propidium iodide (0.1 mg/mL) and Hoechst 33258 (3 μg/mL) in culture medium. After incubation,

cells were washed four times with PBS and analyzed with a fluorescence microscope (Olympus BX51) equipped with filters 365/465 and 530/615. Images were captured and analyzed at single-blind.

DNA strand breaks quantification by TUNEL assay

After treatment with BaP1, cells were fixed in 4% paraformaldehyde for 24 h at 4 °C, washed in PBS and permeabilized in 0.1% sodium citrate, containing 0.1% Triton X-100 for 20 min at 4 °C. Cells were then resuspended in a final volume of 50 µl of TUNEL buffer (0.3 nmol FITC-dUTP, 3 nmol dATP, 50 nmol $CoCl_2$, 5 U TdT, 200 mM potassium cacodylate, 250 µg/mL bovine serum albumin and 25 mM Tris–HCl, pH 6.6) for 1 h at 37 °C. After washing with PBS, cells were analyzed by flow cytometry in an EPIC XL Analyzer (Beckman-Coulter).

Statistical analysis

Results are expressed as means ± SE. Statistical significance was determined by ANOVA test, followed by Tukey HSD pos-hoc. $P<0.05$ was considered to be statistically significant.

Results

Cytotoxicity and loss of adherence induced by metalloproteinase BaP1 in adherent cells

Bovine aortic endothelial cell line (BAEC) was treated with different concentrations of metalloproteinase BaP1 for 24 h to determine its ability to induce cytotoxicity and loss of adherence (Fig. 1). LC_{50} for cytotoxicity was 4.2 µg/mL, reaching maximum values at 50 µg/mL (Fig. 1A). Toxicity kinetics with the maximum concentration tested (200 µg/mL) is displayed in Fig. 1C, showing more than 80% of cell death at 24 h of treatment. 72 h of exposure at different BaP1 concentrations (Fig. 1A) showed an LC_{50} of 1.8 µg/mL.

When BAEC plate detachment was determined at 24 h, it was observed a statistically significant loss of adherence since around 12 µg/mL and the highest effect was observed at 50 µg/mL, reaching about 80% of detachment (Fig. 1B). At that concentration (50 µg/mL) about 15% of cell loss was observed since the first hour of treatment and more than 90% of the effect was reached after 7 h of BaP1 exposure (Fig. 1D).

When cervix adenocarcinoma cell line HeLa was incubated with BaP1 for 24 h, no toxicity was induced (Fig. 2A). Increasing the time of exposure to 72 h, almost no toxicity was also seen, except for a small statistically significant effect at the highest concentration tested: 200 µg/mL (Fig. 2A). Adherence was rapidly lost in this cell line at concentrations as low as 1.5 µg/mL (when the effect started). Highest loss of adherence was observed at 25 µg/mL of BaP1 (Fig. 2B). At this concentration (25 µg/mL) adherence kinetics showed that about 30% of detachment was seen since the first minutes of treatment and after 8 h, detachment was present in almost 90% of the cells (Fig. 2C).

Characterization of cell death mechanisms induced by metalloproteinase BaP1 in BAEC

Since toxicity induced by BaP1 was very high in BAEC and endothelial cells could be the target for the toxin in vivo, we decided to characterize the mechanisms involved in cell death. When we determine DNA fragmentation by PI labeling and flow cytometry, we observed that both HeLa and BAEC were susceptible to suffer apoptosis (Fig. 3). In BAEC, concentrations as high as 200 µg/mL gave DNA fragmentation values of around 12% of the cells at 24 h (Fig. 3B). In the case of HeLa, the values after 24 and 72 h of treatment with 50 µg/mL of the toxin were around 20%.

Since cytotoxicity values induced by BaP1 in BAEC do not seem to correlate with the values of apoptosis, at least with the late sign of this process (DNA fragmentation), we decided to use other approaches to characterize the mechanisms of cell death involved in the process (Fig. 4).

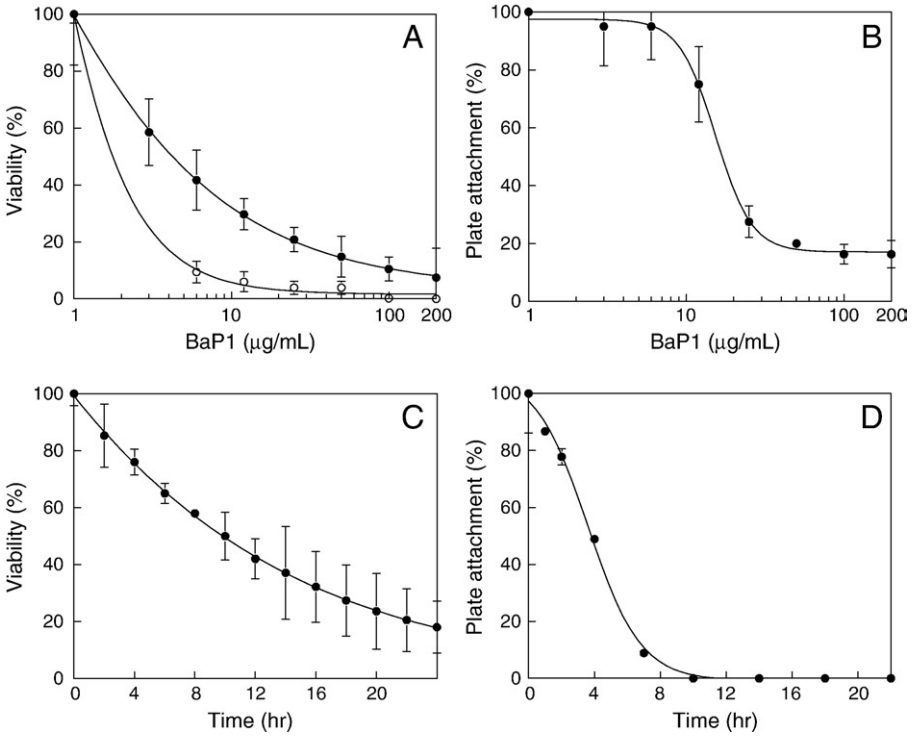

Fig. 1. Cytotoxicity and loss of adherence induced by BaP1 on BAEC. A, B: Cells were incubated with different concentrations of BaP1 for 24 h (●) and 72 h (○). C, D: Cells were incubated with 200 (C) or 50 µg/mL of BaP1 (concentration able to induce maximum adherence loss) (D) for different periods of time. Cytotoxicity was determined by XTT reduction and loss of adherence was quantified by MTT reduction. Values represent the mean ± SE of one representative experiment performed in triplicate.

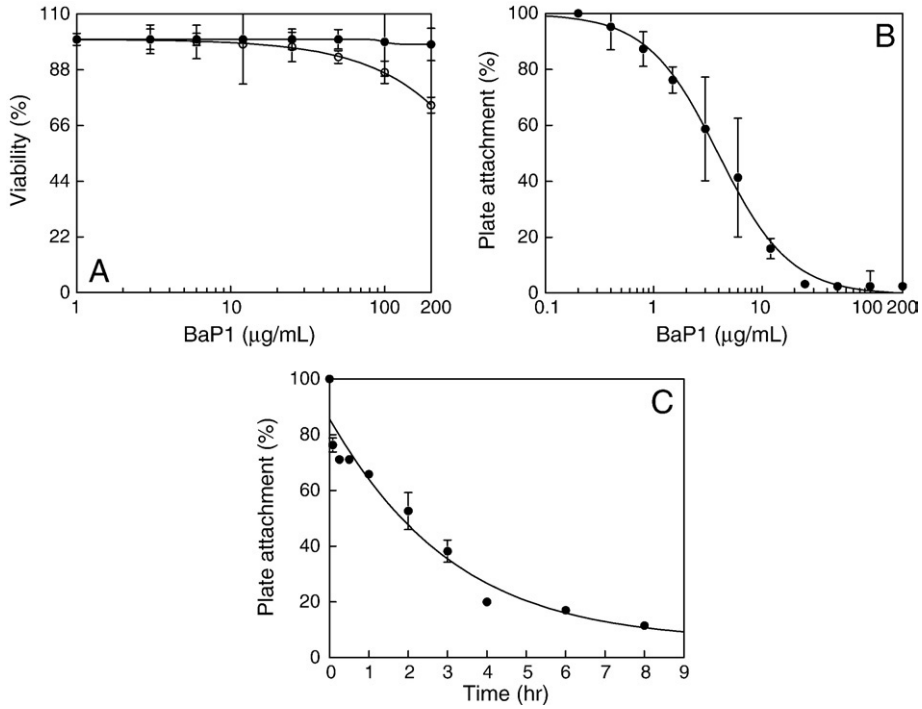

Fig. 2. Cytotoxicity and loss of adherence induced by BaP1 on HeLa. A, B: Cells were incubated with different concentrations of BaP1 for 24 h (●) and 72 h (○). C: Cells were incubated with 25 μg/mL of BaP1 (concentration able to induce maximum adherence loss) for different periods of time. Cytotoxicity was determined by XTT reduction and loss of adherence by calcein uptake. Values represent the mean ± SE of one representative experiment performed in triplicate.

BAEC were labeled with three different fluorochromes to quantify the cells that were in early apoptosis (with condensed chromatin), autophagy (presence of vacuoles) and necrosis (membrane rupture). Fig. 4 shows cells treated with 200 μg/mL of BaP1 and labeled with Hoechst 33258, monodansylcadaverine (MDC) and PI. Treatment resulted in almost 40% of cells with condensed chromatin (increased blue fluorescence, Figs. 4A,B), showing a higher apoptotic value than the one observed when DNA fragmentation was measured by flow cytometry (Figs. 3A,B). It was also observed that a few cells presented membrane disruption, since they show red fluorescence (PI labeling). When cells double-labeled with blue and red fluorochromes were counted, it was evident that most of the cells were positive for both fluorochromes, indicating that probably necrosis was secondary to the apoptotic effect (Figs. 4A,B). However, these results were not statistically significant and if necrosis was present, the effect is probably very small (Fig. 4B).

When autophagic cell death was tried to approach by labeling the presence of vacuoles (green fluorescence) with non-specific MDA staining (Biederbick et al., 1995), we observed that most of the control cells seem to present vacuoles but that these structures decreased after treatment with BaP1 (Fig. 4B). Staining disappeared when control cells were treated with trypsin also (insert in Fig. 4A) which could indicate that cells in suspension present autophagy inhibition, however the results should be interpreted carefully since MDA-labeling has been associated more with late-endosomal and lysosomal markers than with autophagosomes, even when in some cases it has been shown to correlate with autophagic activity (Mizushima, 2004). Even when this result could be ruling out the participation of autophagic cell death as part of the mechanism of BaP1 toxicity on BAEC, when an inhibitor of autophagy (NH_4Cl) was used to try to determine the role of this type of cell death, we observed an inhibition of the cytotoxicity in the presence of the inhibitor (Fig. 4C). LC_{50} in the absence and presence of the inhibitor was 4.5 and 50.3 μg/mL, respectively; suggesting that autophagic cell death could be also present in BAEC treated with this toxin.

To continue with the characterization of the mechanism of cytotoxicity, we did a last experiment using several proteinase inhibitors. Fig. 5 shows that pre-treatment with both caspase and calpain inhibitors induced a 30–40% statistically significant decrease in cytotoxicity induced by BaP1. This confirmed the involvement of apoptosis in the process, but the presence of other types of cell death, could be also occurring.

DNA fragmentation induced by metalloproteinase BaP1 in non-adherent cells

To determine whether BaP1 was toxic for non-adherent cells, we incubated Jurkat cells with BaP1 for 24, 48, 76 and 92 h. At the first 48 h we observed very few late apoptotic cells (with fragmented DNA) at all the concentrations tested, but at longer times, it was clear that BaP1 was able to induce DNA fragmentation in this cell line too (Fig. 6A). The values at 76 h were similar than the ones observed in adherent cell lines, around 15% of DNA fragmentation. At 92 h around 50% of the cells had DNA fragmentation at concentrations of 100 and 150 μg/mL. We confirmed apoptosis by TUNEL, at 96 h of treatment, testing lower concentrations of BaP1 (Fig. 6B).

When we observed the phases of the cell cycle and quantified the number of cells on each phase, we clearly detected a cell cycle arrest in S phase (Fig. 6A), which could explain the apoptotic effect. This effect was not observed in adherent cells at the times tested (Fig. 3), suggesting that even when apoptosis occurs in both types of cells, triggered mechanisms could be different in non-adherent and adherent cell lines.

Discussion

Anoikis is defined as the apoptosis that results in response to inappropriate cell–ECM interactions, and it has been suggested that this process is probably not triggered by a specific stimulus but by a broad range of cellular responses that induce loss of adhesion and that utilize diverse signaling pathways (Gilmore, 2005).

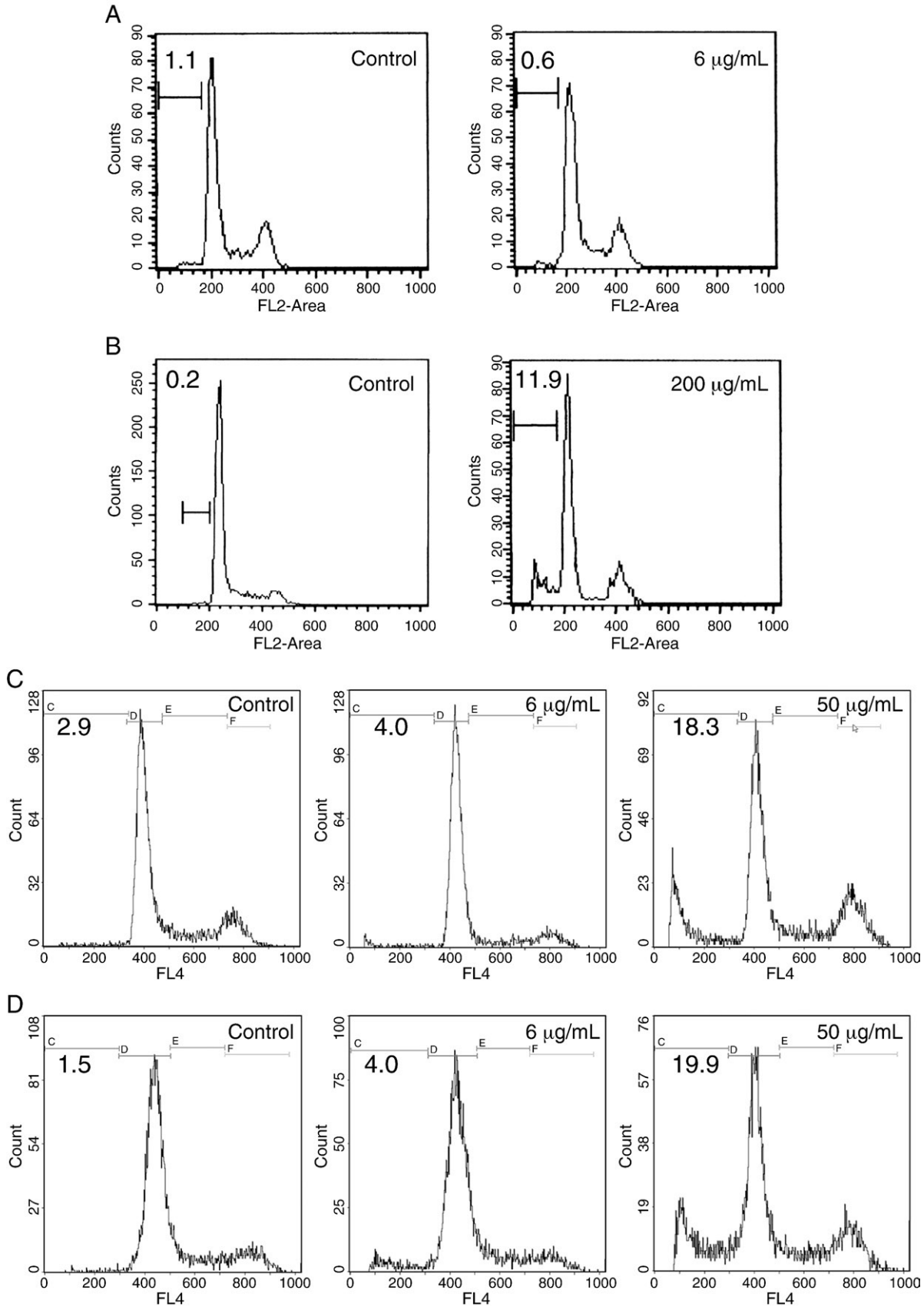

Fig. 3. DNA fragmentation induced by BaP1 on BAEC and HeLa. A, B: BAEC were treated with 6 and 200 μg/mL of BaP1 for 24 h. C, D: HeLa were treated with 6 and 50 μg/mL of BaP1 for 24 (C) and 72 (D) h. Late apoptosis was quantified by flow cytometry after propidium iodide labeling of permeabilized cells. Apoptotic index values are presented in the left corner of each figure. Results show one of three representative experiments.

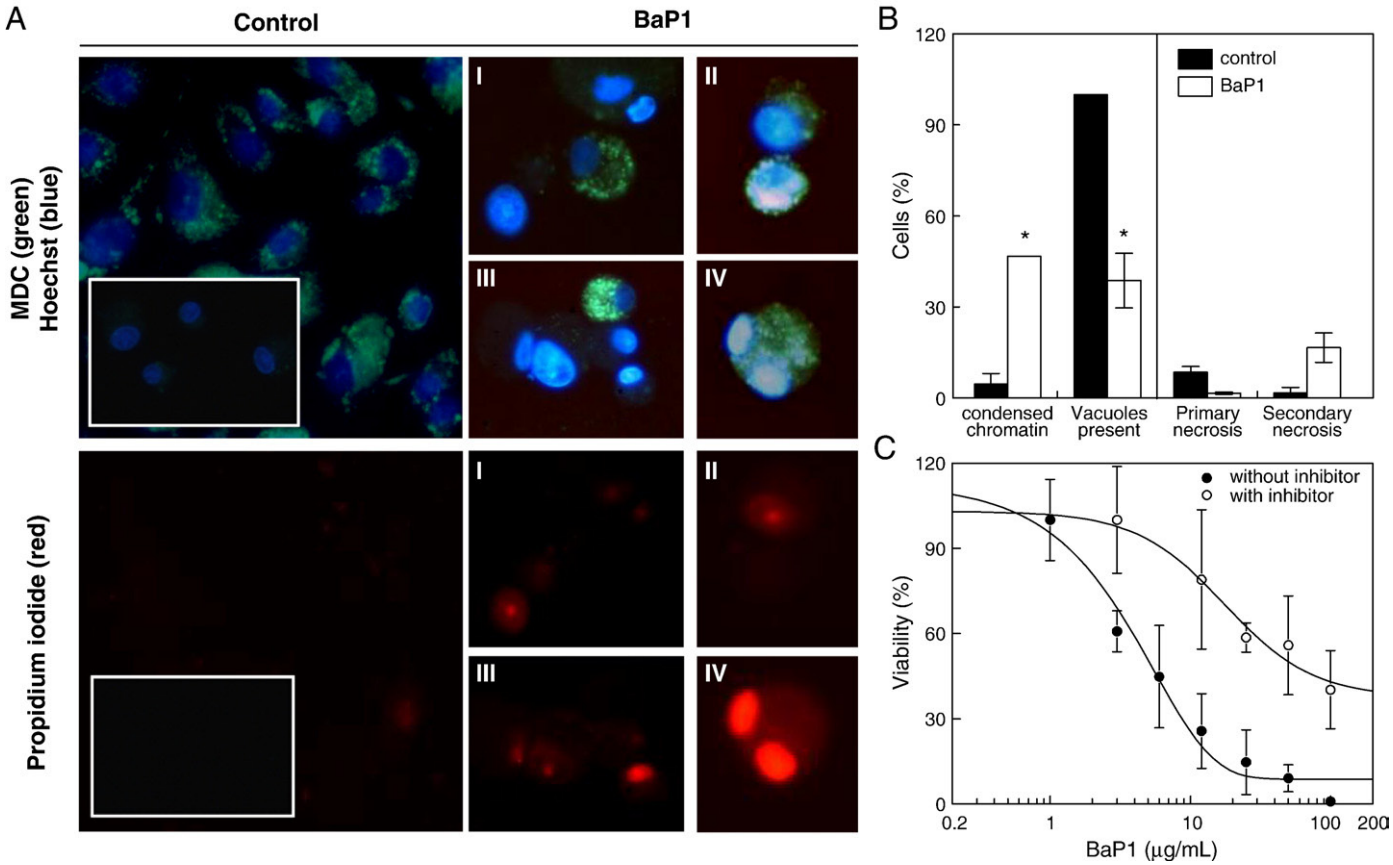

Fig. 4. Chromatin condensation, vacuole presence and membrane rupture determination induced by BaP1 on BAEC. A, B: Cells were incubated with 200 µg/mL of BaP1 for 24 h and stained at the same time with Hoechst and MDC (A, upper panel) and PI (A, lower panel). B: Approximately 200 cells from 10 random fields were counted and results are presented as mean ± SE of two independent experiments. I, II, III, IV are fields chosen randomly to show the same non-permeabilized cells (labeled with three fluorochromes) under the two different fluorescence filters used here. Insert indicates a population of cells incubated with trypsin as a control of loss of adherence. C: Cells were treated with different concentrations of BaP1 in absence of presence of autophagy inhibitor NH_4Cl. Cytotoxicity was measured by XTT reduction and results represent the mean ± SE of one representative experiment performed in triplicate. *Statistically significant differences compared to BaP1 treatment (ANOVA and Tukey's test, $p<0.05$).

We have previously shown that, like other SVMPs, BaP1 is able to induce early apoptosis in endothelial cells, probably through the extrinsic pathway (Díaz et al., 2005). Some articles have hypothesized that the mechanism of apoptosis induced by SVMPs could be anoikis (Tanjoni et al., 2005), but the evidence for concluding that is only circumstantial. Therefore, we decided to test whether loss of attachment and cell death induced by metalloproteinase BaP1, were two related processes or whether apoptosis could be induced regardless of cell attachment, as for example, in non-adherent cells. To test that, we used bovine aortic endothelial cells (BAEC) and cervix adenocarcinoma (HeLa).

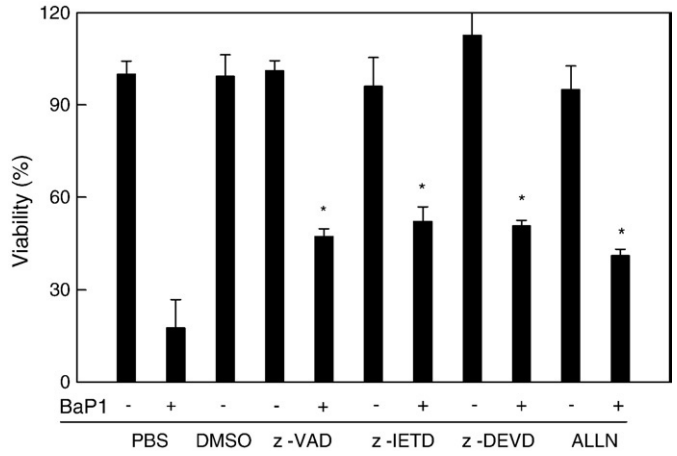

Fig. 5. Effect of different protease inhibitors on cytotoxicity induced by BaP1 on BAEC. Three caspase inhibitors (pan-caspases, caspase-3 and caspase-8) and one calpain inhibitor were pre-incubated with the cells for 2 h at a concentration of 30 µM and cells were incubated with 200 µg/mL of BaP1. Cytotoxicity was measured by XTT reduction. Values represent the mean ± SE of one representative experiment performed in triplicate. *Statistically significant differences compared to BaP1 treatment (ANOVA and Tukey's test, $p<0.05$).

Both adherent cell lines were not equally sensitive to BaP1, in fact, HeLa were practically resistant to the treatment. Results with 3T3 (not shown) indicate that fibroblasts are partially resistant to BaP1, and it has been observed that they are even able to survive when ECM is absent (Gilmore, 2005). In the case of detachment and cell death induced by SVMPs, several studies have also shown that endothelial cells are more sensitive to suffer apoptosis than fibroblasts (Tanjoni et al., 2005; Krieger de Moraes and Salistre-de-Araujo, 2006). However, it is difficult to compare between cell lines, since not all SVMPs induce death at the same time and concentrations, and some of the conditions of the experiments reported in the literature are different, as for example, the presence or absence of fetal bovine serum, which is relevant because it contains some metalloproteinase inhibitors (Anai et al., 1998; Ribeiro Filho et al., 2003).

In the case of SVMP jararhagin, for instance, the apoptotic effect induced on endothelial cells is observed at 72 h of treatment (Tanjoni et al., 2005), which indicates that even in these cells (tEnd), which are expected to be more sensitive, the effect induced by some SVMP occurs late. In a previous study on endothelial EA.hy.926, our group observed that cells were suffering apoptosis very early, since the first hours of treatment (Díaz et al., 2005). Some studies (Obrig et al., 1993) had already shown cell-type differences to the exposure to

these enzymes, given that endothelial cells were more sensitive to *Crotalus rubber* metalloproteinases than Vero cells, for instance. However, not all SVMPs induce apoptosis in endothelial cells; Obrig et al. (1993) showed that *Crotalus atrox* atroxase was not cytotoxic towards HUVEC, even after 72 h of treatment, whereas Araki et al. (1993) had previously observed that whole *C. atrox* venom did induce apoptosis in these endothelial cells. The reference does not mention, however, whether atroxase was able to induce cell detachment of HUVEC. Another factor that makes difficult the comparison between cells is the fact that different approaches to determine cytotoxicity and apoptosis give different results. In this work we observed that even when BAEC seem to suffer very high cytotoxicity, when DNA fragmentation was determined, the effect was very low. Our results indicate that other mechanisms of cell death could be involved and late indicators of apoptosis such as DNA damage, are sometimes misleading, since apoptosis is occurring even when DNA is still intact.

Since DNA fragmentation is a very late event in apoptosis and does not strictly correlate with cytotoxicity, we used another approach to test whether apoptosis was taking place and if the mechanism was triggered by extrinsic or intrinsic pathways. So, we decided to quantify the cytotoxic

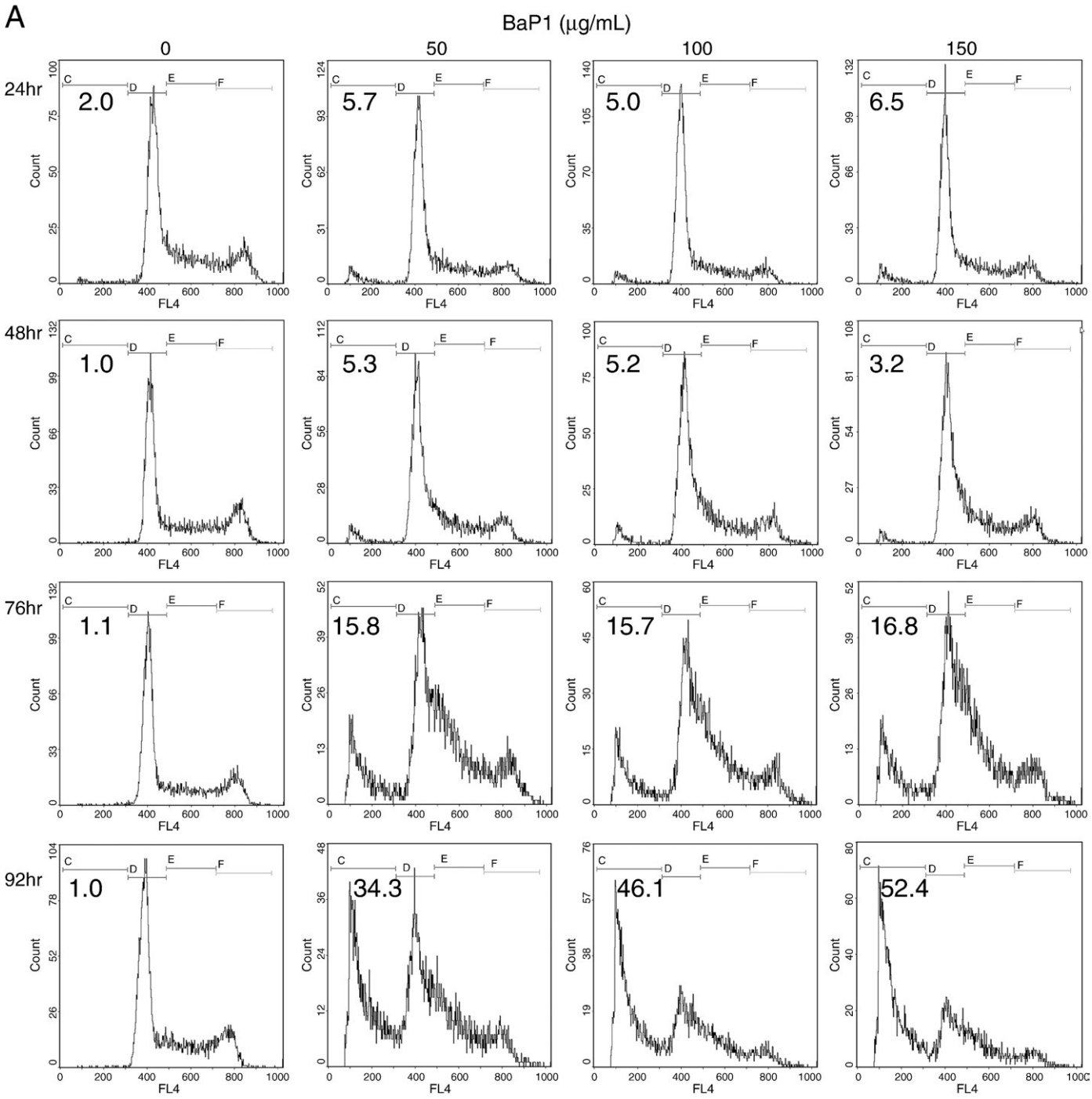

Fig. 6. DNA fragmentation induced by BaP1 on Jurkat cells. A: Cells were treated for 24, 48, 76 and 92 h with different concentrations of BaP1 and late apoptosis was determined by flow cytometry after PI labeling of permeabilized cells. Values of DNA fragmentation are presented in the left corner of each figure. Results show one of three representative experiments. B: Jurkat cells were treated for 96 h with BaP1 at different concentrations and DNA fragmentation was determined by TUNEL. Thapsigargin was used as positive control of apoptosis (insert). Results show one of three representative experiments.

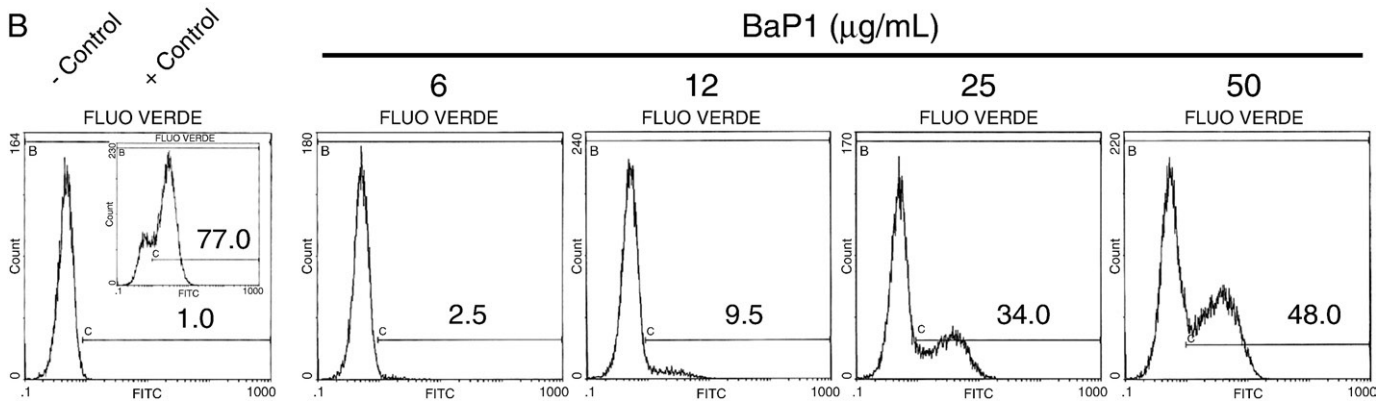

Fig. 6 (*continued*).

effect in the presence of several protease inhibitors. The analysis in the presence of caspase inhibitors corroborated apoptosis, since Z-VAD-FMK (a pan-caspase inhibitor) significantly decreased BaP1 induced cytotoxicity. In agreement with our previous results in EA.hy.926 endothelial cells (Díaz et al., 2005), apoptosis could be triggered by extrinsic pathway since either inhibitors of caspase-8 or -3 (Z-IETD-FMK and Z-DEVD-FMK) also decreased cytotoxicity induced by the toxin. Additionally, it is known that during apoptosis moderate increases in cytosolic Ca^{2+} could occur and that other proteinases (calpains) could be activated (Liu et al., 2004; Saez et al., 2006). The effect of the inhibitor of neutral cysteine-proteinases ALLN, indicates that other proteases could also be participating in BaP1-induced cytotoxicity.

When we tried to determine whether autophagy was involved in the mechanism of cell death induced by BaP1, we labeled the cells with MDA, a fluorescent staining that had been proposed as a tracer for autophagic vacuoles (Biederbick et al., 1995). However, more recently, it has been indicated in the literature (Mizushima, 2004) that MDC could probably bind late-endosomal and lysosomal compartments and its specificity for autophagosomes has been questioned. Our results showing that control cells have more positive MDC staining than BaP1 treated cells, indicates that probably from these results we cannot conclude autophagy is taking place. However, when we treated the cells with inhibitor of autophagy NH_4Cl, we observed an inhibition of the cytotoxicity, which could indicate the participation of this mechanism of cell death.

A study performed by Wan et al. (2006) showed that bladder carcinoma ECV-304, previously thought to be from endothelial origin, were resistant to a SVMP from *Trimerresurus stejnegeri* (TSV-DM). The article is interesting because it shows that the toxin can induce inhibition of cell proliferation. In our study we observed cell cycle arrest in S phase when Jurkat cells were treated with BaP1, and we suggest that the apoptotic effect induced on these cells, was probably the result of cell cycle inhibition. We also observed that ECV-304 were partially resistant to BaP1 (results not shown).

Wu and Huang (2003), in a very complete study where they characterized the effect of SVMP graminelysin on endothelial cells, had previously suggested that, according to their results, the apoptotic effect induced by the proteinase was not anoikis. They based their conclusion on examination of cells that were detached and the ones that remained anchored, both showing signs of apoptosis such as DNA fragmentation and PARP cleavage. In other words, they demonstrated that graminelysin caused endothelial apoptosis prior to cell detachment. However, this raises the question of whether cleavage of some specific points of attachment (perhaps some contacts with survival-related integrins) that do not completely remove the cells from the substratum, could be enough to induce apoptosis. This could still imply a mechanism of death related to anoikis, even though cells remained somehow attached, since according to the definition of anoikis, loss of ¨appropriate¨ attachment has to be happening to interfere with survival. Gilmore (2005) pointed out that in many experimental systems, the study of anoikis is carried out in completely ECM-detached cells, which is probably uncommon in in vivo conditions, at least for endothelial cells, and that the wrong type of connections with ECM can have the same consequences as no connections at all. Also, Chen et al. (1997) showed that cell survival can be achieved with very few points of ECM attachment but if they could spread and have certain degree of extension.

In this study we did not found a correlation between plate detachment and cell death triggered by BaP1 in endothelial cells, suggesting that BaP1 could be inducing some degree of cytotoxicity by a mechanism independent of anoikis. If the process induced by SVMPs is not anoikis, there are several possibilities that could explain the apoptotic effect observed in this study. Metalloproteinases have many substrates other than the ECM components, which could have a profound influence in cell behavior, survival and death. In the case of MMPs, they cleave soluble molecules and cell surface receptors such as several cytokines and growth factors (Li et al., 2007). Moreover, when they induce partial proteolysis of ECM molecules, can generate biologically active fragments known as matrikines, which are able to regulate apoptosis (Mannello et al., 2005). Another interesting possibility is the direct interaction of MMPs with integrins, which has been observed in the case of collagenase-1 for integrins $\alpha1\beta2$, $\alpha v\beta3$ and $\alpha5\beta1$, and these interactions could be involved in regulation of biological activities, including apoptosis (Mannello et al., 2005). Since some snake venoms and SVMPs can activate MMPs (Rucavado et al., 1995; Wu and Huang, 2003; Saravia-Otten et al., 2004), both kinds of proteinases together could be responsible for the effects observed in cells.

On the other hand, both MMPs and SVMPs have been shown to generate active soluble cytokines such as FasL and TNFα. For instance, matrilysin (MMP-7) is able to generate active soluble FasL and trigger apoptosis in epithelial cells (Powell et al., 1999). On the other hand, jararhagin is able to cleave soluble TNFα in vitro (Moura da Silva et al., 1996; Clissa et al., 2001).

In conclusion, this work suggests that BaP1 induces apoptosis in BAEC and in a lesser extent in HeLa, but at least endothelial cells apparently suffer other types of cell death that generate the high cytotoxic values observed by XTT reduction. Our results demonstrate that early apoptosis could be occurring without DNA fragmentation present, since chromatin condensation is occurring and caspases are active and responsible of part of the cytotoxicity. Also, we showed that cell death could not be the result of anoikis, since cells that remain in suspension and do not have a strong contact with ECM (Jurkat), are able to die by apoptosis too. This is strengthened by the fact that in adherent cells there is no correlation between detachment and loss of viability, since 50% of cytotoxicity can be reached without any detachment.

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