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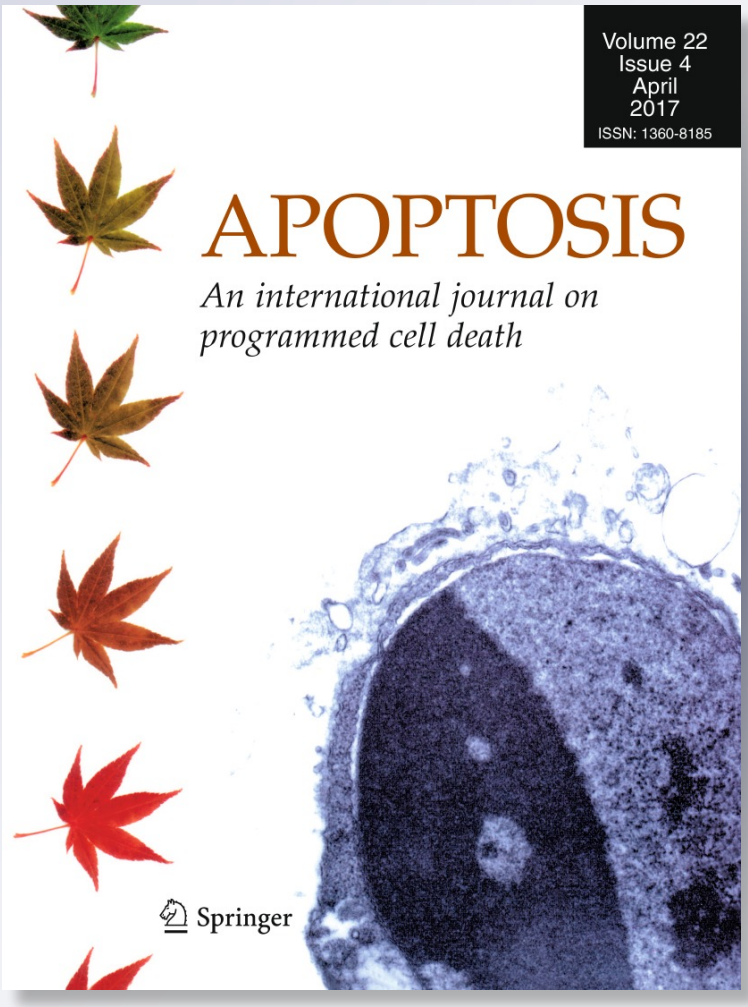

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Apoptosis induced by a snake venom metalloproteinase from *Bothrops alternatus* venom in C2C12 muscle cells

Soledad Bustillo[1] · Andrea C. Van de Velde[1] · Verónica Matzner Perfumo[1] · Claudia C. Gay[1] · Laura C. Leiva[1]

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Abstract In this study, the apoptosis inducing effects of baltergin as well as its influence on cell adhesion and migration on muscles cells in vitro were studied. Morphological analysis made by scanning electron and phase contrast microscopy demonstrated typical futures of programmed cell death, apoptosis. This mechanism was confirmed by fluorescence staining, molecular analysis of endonuclease activity and increased mRNA expression level of two representative genes (p53 and bax). On the other hand, baltergin exert an inhibition effect on myoblast cell adhesion and migration in vitro probably through a mechanism that involves the interaction of this enzyme with cell integrins. In conclusion, our results suggest that the absence of appropriate extracellular matrix contacts triggers anoikis. Therefore, this is the first report that demonstrated the mechanism of programmed cell death triggered by baltergin, a PIII metalloprotease isolated from *Bothrops alternatus* venom, in a myoblast cell line.

Keywords Baltergin · Metalloproteinase · *Bothrops alternatus* · Anoikis · Apoptosis

Introduction

Snake venom metalloproteinases (SVMPs) play a relevant role in the pathogenesis of *Bothrops* intoxication. The functional diversity of SVMPs is in part due to the structural organization of different combinations of catalytic, disintegrin, disintegrin-like and cysteine-rich domains, which categorizes SVMPs in 3 classes of precursor molecules (PI, PII and PIII) further divided in 11 subclasses. This heterogeneity is currently correlated to genetic accelerated evolution and post-translational modifications [1].

According to proteomic studies, SVMPs consists about 43% of *Bothrops alternatus* venom [2]. Baltergin is a member of P-III class of SVMPs isolated from this venom which is responsible to induce relevant local effects such as hemorrhage, edema and myotoxicity as well as systemic bleeding in mice [3]. Previous studies demonstrated cytotoxic and apoptotic effects induced by *B. alternatus* whole venom on muscle C2C12 cell line [4]. In particular, this metalloproteinase together with a non-toxic acidic PLA_2 isolated from the same venom, evidenced a synergistic effect on cell detachment of the mentioned muscle cell line and an endothelial cell line (tEnd) [5, 6]. This loss of adhesion from culture substrate, occurred without involving a cytolytic effect, as indicated by the lack of release of cytosolic LDH to supernatants in cells exposed to the toxins.

The use of skeletal muscle myoblasts/myotubes from C2C12 cell line as targets for venom toxins has been proposed as a useful in vitro model to study their myotoxic mechanism(s), as it correlates with the muscle-damaging activity observed in vivo [7]. Therefore, this cell culture is an appropriate cell model system to evaluate the myotoxicity caused by snake venoms or isolated toxins as proven by several studies [4, 8–13].

In anchorage-dependent cells, cell-to-cell and cell-to-matrix contacts are necessary for the maintenance of cell survival [14]. Linked with cell adherence status, anoikis has been described as a specific type of apoptosis caused by inappropriate cell-ECM interactions and all of the features that characterize apoptosis, including nuclear fragmentation, membrane blebbing and externalization of

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phosphatidyl serine (PS), are observed during this process [15].

It has been reported earlier that different snake venom metalloproteases (SVMPs) induce apoptosis. In particular, class PIII of these enzymes has demonstrated to induce this type of cell death principally on endothelial cells as a consequence of loss of adhesion [16–20]. However, the mechanism of cell death triggered by baltergin was not yet studied.

Thus, in order to achieve a better understanding on the role of metalloproteinase activity in envenomation, we analyzed the events involved in cell death induced by baltergin on muscle cells and its pharmacological potential as a structural model for drug design for cancer therapy.

Materials and methods

Venom and toxin

B. alternatus crude venom was supplied by the Serpentarium of Corrientes, Argentina and kept at −20 °C after vacuum dried. The metalloproteinase, baltergin was isolated from *B. alternatus* venom as described by Gay et al. [3]. In order to assay the isolated enzyme for purity, electrophoresis was performed on 12% polyacrylamide slab gels following the method of Laemmli [21].

Cell culture

C2C12 (CRL-1772, ATCC) is a subclone murine skeletal muscle cell line derived from mouse myoblast cells obtained from normal adult C3H leg muscle. This cell line rapidly differentiates and produces extensive contracting myotubes that express characteristic muscle proteins [22]. Cytolysis was assessed in undifferentiated myoblasts. Cells were grown in 25 cm^2 flasks using Dulbecco's minimum essential medium (DMEM—Gibco Argentina), supplemented with 5% fetal bovine serum (FBS—Natocor Argentina), Penicillin–Streptomycin (Invitrogen) in a humidified atmosphere with 5% CO_2 at 37 °C.

Phase contrast microscopy (PCM)

C2C12 cells were seeded in 6-well plates, 1×10^6 cells per well in the same growth medium (DMEM-FBS 5%). When monolayers reached 80–90% confluence, culture medium was washed out and 200 µg/mL of the purified enzyme was added (3 mL/well). After 4 h incubation, at 37 °C and 5% CO_2 in humid atmosphere, the cell morphological changes were investigated qualitatively using a phase contrast microscope (Axiovert 40®, Carl Zeiss Argentina). The photos were taken with a digital camera (Canon CCD 2272 × 1704, Argentina). In addition, the effect of baltergin inhibited catalytically with Na2EDTA was assayed. Briefly, 100 µL of enzyme (4 mg/mL) was preincubated with the same volume of Na2EDTA (100 mM) for 1 h at 37 °C. Excess reagent was eliminated by gel filtration on Sephadex G-25. The inhibited fraction, baltergin-Na2EDTA, was collected and protein concentration was determined by Bradford and adjust to 200 µg/mL [23]. Moreover, the effectiveness of inhibition was checked before the exposure to cells by the determination of the residual proteolytic activity [24].

Scanning electron microscopy (SEM)

Cells were grown on coverslips until 70–80% confluence and then incubated with PBS (control) or baltergin (200 µg/mL) during 4 h at 37 °C. Cells were then fixed at room temperature for 60 min in 2.5% glutaraldehyde (pH 7.4). Coverslips were air dried and dehydrated with increasing concentrations of acetone, dried by the CO_2 critical-point method, sputtercoated with gold/palladium and examined with a JEOL 5800 LV Scanning Electron Microscope (SEM).

Fluorescence staining

Myoblast cells were grown on cover slips and treated with baltergin metalloproteinase (200 µg/mL) for 4 h at 37 °C. PBS was used instead of baltergin for control assays. After incubation, cultured myoblasts were washed twice with PBS and gently mixed with a mixture of acridine orange (AO) (1 µg/mL) and ethidium bromide (EB) (1 µg/mL) dye solution for one minute as described by Spector et al. [25]. Coverslips were applied to the slides; afterwards, the sections were observed and photographed under a fluorescence microscope (Axioskop 40®/Axioskop 40 FL®, Carl Zeiss, Argentina).

Adhesion assay

For adhesion assay, C2C12 cells ($2.5–3 \times 10^4$/well) were preincubated for 30 min at room temperature with Baltergin (10–300 µg/mL) or culture medium (control group) and then added to 96-well plate previously coated with FBS:PBS (1:4) for 1 h at 37 °C followed blocked with 1% heat-inactivated BSA (Sigma-1 µg/µL) in PBS. After 1.5 h, non-adherent cells were removed by careful aspiration and washed with PBS. Adherent cells were fixed with methanol:glacial acetic acid (3:1 ratio), and stained with 0.5% crystal violet in 20% (v/v) methanol. After dye release, adherent cells were microscopically observed and recorded photographically. The dye was released from the cells by addition of ethanol:glacial acetic acid (3:1 ratio).

The optical density of the released dye solution was read at 620 nm and the percentage of cell adhesion was determined by comparing the resulting absorbances with the mean absorbance of control groups (considered as 100% of adhesion) [26]. For the assessment of cytolysis, the release of the cytosolic enzyme lactate dehydrogenase (LDH) was used as an indicator of membrane damage [5]. Aliquots (40 µL) of the supernatant in culture wells were collected, and LDH activity was determined by using a UV kinetic assay kit (LDH BR CE, Spain). Cytolysis was expressed as percentage, using as 0% and 100% reference values the LDH activity of supernatants from cells exposed to medium alone, or to 0.1% (v/v) Triton X-100, respectively. All assays were carried out in triplicate wells.

Migration assay

Cell migration was measured by Wound-healing assay. Briefly, cells were grown to 90% confluence in a 6-well plate at 37 °C, 5% CO_2 incubator. A wound was created by scratching cells with a sterile pipette tip, cells were washed twice with PBS to remove floating cells and then incubated in culture medium in the absence or presence of baltergin (25 and 50 µg/mL) for 24 h. Also, the effect of the same concentrations of enzyme but catalytically inhibited with Na_2EDTA were evaluated. The inhibition process was performed as it was previously described in item 3. Cell migration into the wound surface was determined under an inverted microscope. Wound widths were measured with Image-Pro software and percentage of cell migration was calculated by comparing with control groups (considered as 100% of migration).

DNA fragmentation assay

For the detection of apoptotic DNA cleavage, 1×10^6 cells were grown on 6-well plates at 37 °C, 5% CO_2. Cells were treated with baltergin (200 µg/mL) for 4 h and then trypsinized and lysed with lysis buffer (Tris–HCl 50 mM, pH8.0, EDTA 10 mM, SDS 0.5%, proteinase K 0.5 mg/mL) for 20 min. DNA extraction was made with phenol/chloroform/isoamyl alcohol solution (25:24:1), precipitated with 0.1 volumes of sodium acetate (3 M) and 2.5 volumes of ethanol (96%) and resuspended in buffer Tris–HCl 10 mM pH 8.0, EDTA 1.0 mM pH 8.0 with RNase (20 mg/mL Invitrogen). DNA was visualized by electrophoresis on a 1.5% agarose gel (Biodynamics) containing ethidium bromide (Promega).

RNA isolation and RT-PCR

To determine the expressions of p53 and bax mRNAs, 1×10^6 cells were grown on 6-well plates at 37 °C, 5% CO_2 and treated with baltergin (200 µg/mL) for 4 h. Total RNA was isolated from the cells using Trizol reagent (Invitrogen) according to manufacturer's instructions. Two micrograms of RNA, 0.5 µL of Oligo dt (Biodynamics) and 9.5 µL of dimethyl pyrocarbonate-treated water (DEPC-H_2O) were added together, and the mixture was heated at 70 °C for 5 min. One microliter of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Promega), 5 µL of M-MLV RT buffer (Promega), 1.125 µL of dNTPs (10 mM) (Fermentas), 0.625 µL de RNasa Out (Invitrogen) were then added to the mixture, and the final volume was adjusted to 25 µL with the DEPC-H_2O. Subsequently, the reaction mixture was incubated at 42 °C for 1 h. PCR amplification was performed in a 25 µl reaction volume containing 3 µL of the appropriate cDNA, 2 µL of dNTPs (Fermentas), 1 µL of the 10 µM primer set (Fermentas), 0.12 µL of GoTaq® DNA polymerase (5 unites/µL) (Promega), 5 µL de buffer of reaction buffer (Green GoTaq® buffer) (Promega), 1 µL of Cl2Mg (Promega) and 12.88 µL of H2O. The sequences of mouse p53 primers were 5′-GATGACTGCCATGGAGGAGT-3′ (a 20-mer sense oligonucleotide) and 5′-CTCGGGTGGCTCATAAGGTA-3′ (a 20-mer anti-sense oligonucleotide). The sequences of mouse bax primers were 5′-AGATGAACTGGATAGCAA TATGGA-3′ (a 24-mer sense oligonucleotide) and 5′-CCA CCCTGGTCTTGGATCCAGACA-3′ (a 24-mer anti-sense oligonucleotide). The sequences of the internal control cyclophilin primers were 5′-ACCCCACCGTGTTCTTCG AC-3′ (a 20-mer sense oligonucleotide) and 5′-CATTTG CCATGGACAAGATG-3′ (a 20-mer antisense oligonucleotide). The expected sizes of the PCR products were 664 bp for p53, 270 bp for bax, and 291 bp for cyclophilin. The PCR procedure was carried out using a thermocycler (Thermo) under the following conditions: initial denaturation at 94 °C for 5 min, followed by 25 amplification cycles, each consisting of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s, followed by additional extension step at the end of the procedure at 72 °C for 5 min. Amplification products were separated on 1.5% agarose gel (Biodynamics) and visualized by ethidium bromide staining (Promega). Finally a photographic record of the amplified products was obtained and bands were analyzed by densitometry with computer software Uvi Pro gel documentation system (UV Tech) to calculate relative gene expression.

Statistical analysis

Data represent the mean ± standard deviation (SD) of at least four replications. Statistical significance was tested by one-way ANOVA and Tukey (HSD) and p-values inferior to 0.05 were considered significant.

Results

Phase contrast microscopy (PCM)

Cell morphology analysis by PCM showed that untreated C2C12 cells were homogeneously distributed on cultured field; they exhibited normal, elongated and tapered morphology (Fig. 1a). After 4 h incubation with 200 µg/mL of baltergin cell rounding, chromatin condensation and some areas devoid of cells were observed (Fig. 1b). The inhibition of catalytical activity by Na_2EDTA decreased the number of cells with morphological futures of apoptosis but not abolished completely the effect (Fig. 1c).

Scanning electron microscopy (SEM)

To assess the baltergin-induced change in cell morphology, cells were examined by scanning electron microscopy. As shown in Fig. 2b, c, d, numerous membranes blebbings, decrease in cells density, shrinkage of cell volume, apoptotic bodies and dispersed aggregates of the supernatant were induced with 200 µg/mL of this toxin.

Fluorescence staining

In order to determined whether baltergin induced morphological alterations that could be attributed to an apoptotic mechanism, myoblast cells were incubated with 200 µg/mL for 4 h and then stained with the nucleic acid-binding fluorochromes, acridine orange and ethidium bromide. Control untreated cells exhibited a green fluorescence, due to exclusion of ethidium bromide but not of acridine orange. Viable cells showed a light green nucleus with intact structure and presented punctuate orange red fluorescence in the cytoplasm, representing lysosomes stained by acridine orange (Fig. 3a). After 4 h of incubation with Baltergin, typical features of apoptosis were observed. Apoptotic cells exhibited a bright green nucleus (showing condensation of chromatin), dense green areas and evident membrane blebbing (Fig. 3b).

Adhesion assay

Baltergin (10–300 µg/mL) inhibited cellular adhesion in a concentration-dependent manner (Fig. 4), being significantly different from the control at all concentrations tested. The highest amount of enzyme (300 µg/mL) preincubated with myoblast cells, inhibited cell adhesion by 32% with respect to control assays. This effect was not related to cytolysis, since no significant LDH release to supernatants was detected even at the highest concentration tested. Thus, the enzyme induced endothelial cell detachment without cytolysis, i.e. without plasma membrane disruption.

Migration assay

Wound healing assay was performed to evaluate the inhibitory effect of baltergin on the migratory features of C2C12 cells. The wound scratch in control cells was almost completely closed after 24 h of incubation (Fig. 5b). However, treatment with 25 and 50 µg/mL baltergin resulted in the suppression of wound healing in a concentration-dependent manner, decreasing respect to controls by 15 and 48% respectively (Fig. 5c, e, g). This inhibitory effect on migration was found to be inferior with the catalytically inhibited enzyme, compared with the observed with baltergin alone, for both concentrations assayed (Fig. 5d, f, g).

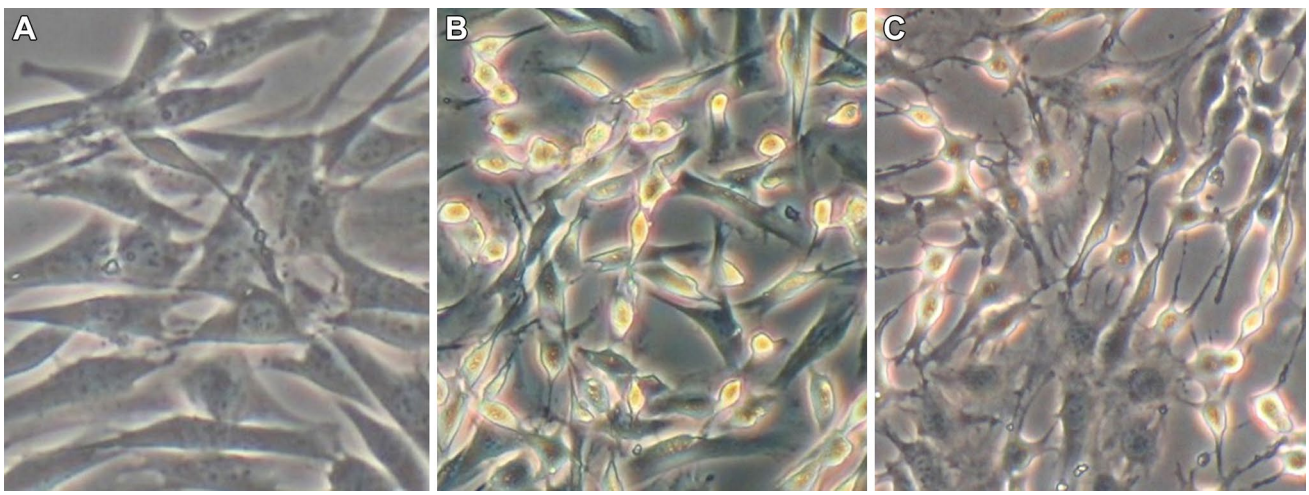

Fig. 1 Morphological changes in myoblast cell cultures under phase contrast microscopy **a** Control, **b** Baltergin, 200 µg/mL for 4 h, **c** Baltergin-Na_2EDTA, 200 µg/mL for 4 h

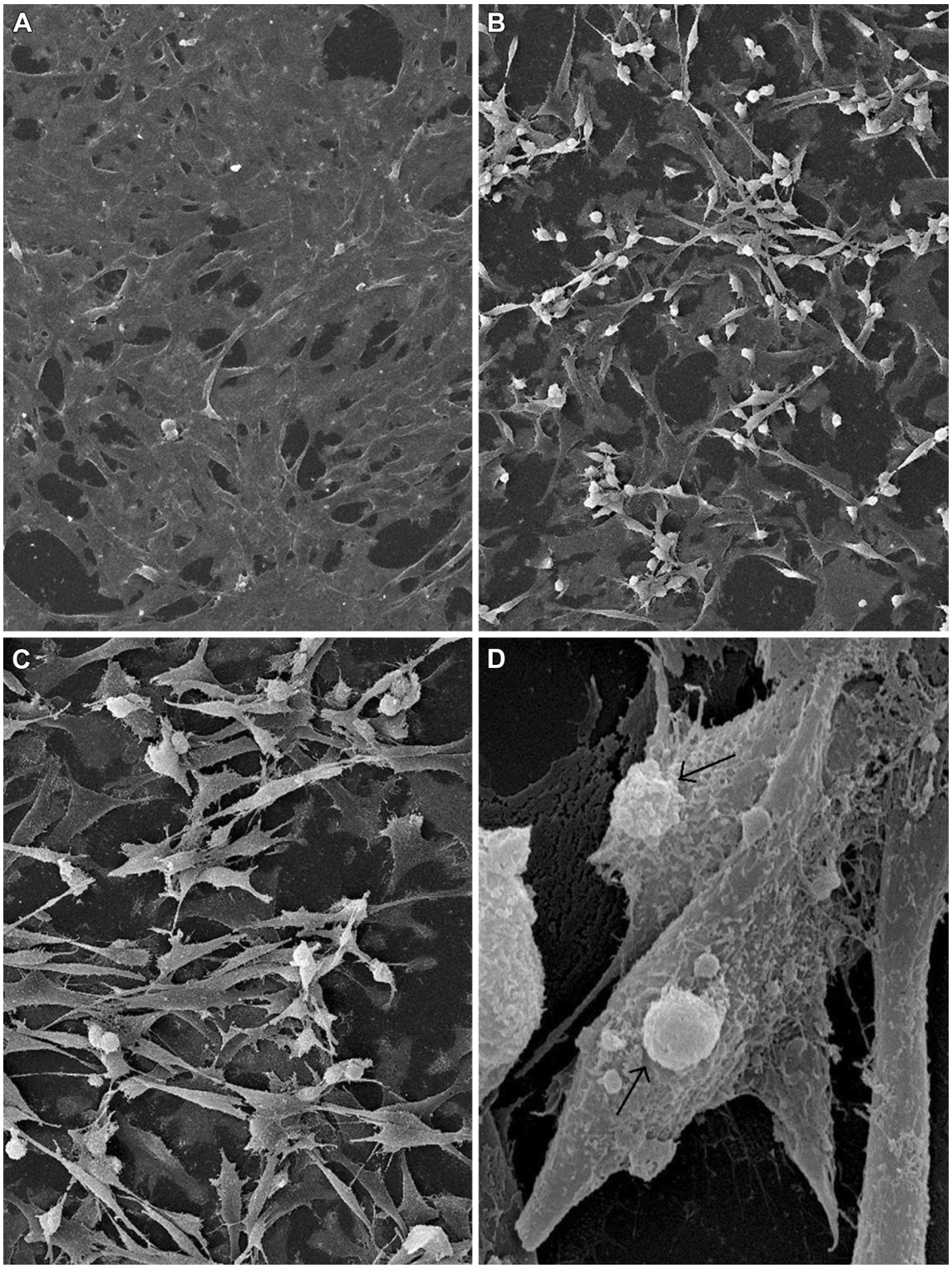

Fig. 2 C2C12 cells observed at SEM after baltergin treatment (4 h). **a** Control myoblasts **b** Baltergin 200 µg/mL (×270) **c** Baltergin 200 µg/mL (×500) **d** Baltergin 200 µg/mL (×4500). Apoptotic bodies (*arrows*)

DNA fragmentation assay

To ascertain that baltergin induces apoptosis, DNA fragmentation that reflects the endonuclease activity was analyzed. As shown in Fig. 6, the treatment for 4 h with 100 µg/mL of enzyme showed fragmentation of DNA but

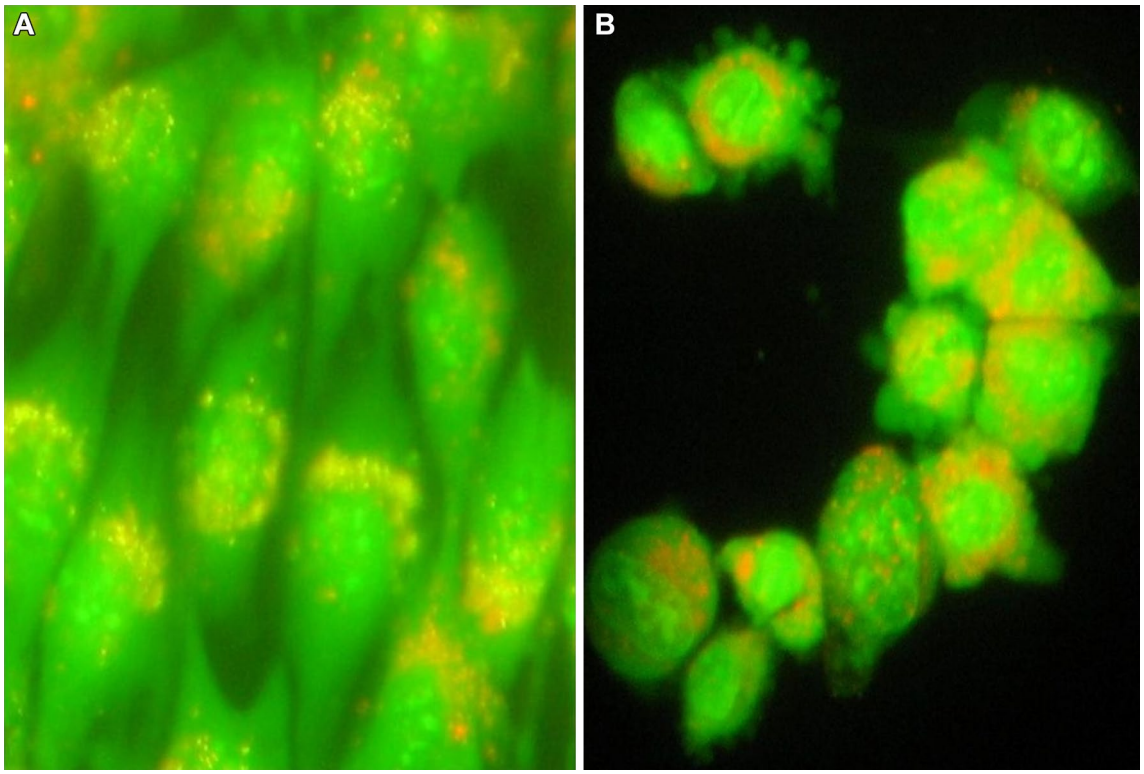

Fig. 3 Acridine orange and ethidium bromide fluorescence staining. **a** Control **b** Representative photomicrograph showing cell shrinkage, chromatin condensation and the formation of blebs on the cell surface associated with apoptosis. Myoblasts were grown on coverslides and treated for 4 h minutes with 200 µg/mL of baltergin

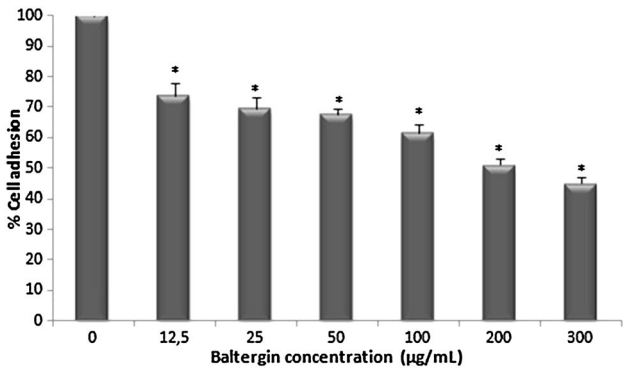

Fig. 4 Effect of baltergin on C2C12 cells adhesion. Myoblasts were added to culture wells precoated with FBS:PBS (1:4) in the presence or absence of baltergin (12.5–300 µg/mL). After 1.5 h incubation, the non-adherent cells were washed away and the adherent cells were stained with Cristal Violet. Cell adhesion is expressed as % of the untreated control. *Each bar* shows the mean ± SD of three independent experiments that were performed in triplicate. *$p < 0.05$ compared with the control

incubation with 200 µg/mL resulted in the formation of fragments that could be seen as the characteristic ladder pattern by electrophoresis.

Effect of baltergin on the expression of p53 and bax mRNA

In the present study, we investigated the mRNA expression level of two representative genes (p53 and bax) after treatment with baltergin using the RT-PCR technique. The copy number of each gene was normalized to the reference gene (cyclophilin) to give relative expression levels, and the change in gene expression was compared with the untreated control samples (Fig. 7a). Our results demonstrated that the mRNA expression level of both genes, increased significantly following treatment with the enzyme as compared to control cells. In particular, levels of bax and p53 mRNA following the treatment with 200 µg/mL for 4 h were increased to 1.19 and 1.51 respectively (Fig. 7b, c).

Discussion

Snake venom metalloproteinases (SVMPs) degrade various components of the basement membrane and are also able to hydrolyze cell membrane proteins, such as integrins and cadherins, involved in cell–matrix and cell–cell adhesion [27]. Molecular approaches have been previously

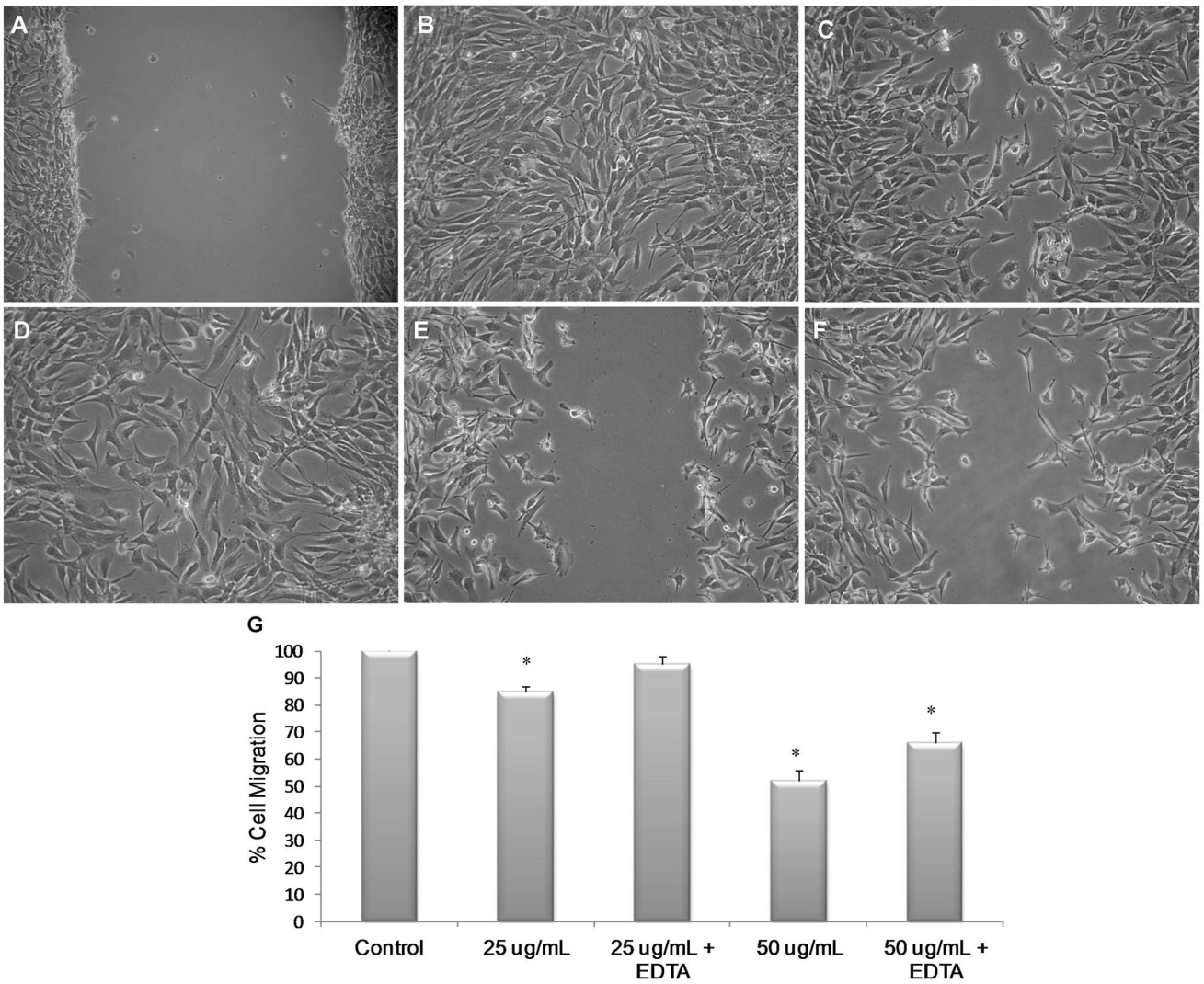

Fig. 5 Effect of baltergin on migration in C2C12 myoblast cells. **a–d** Photographs of wounds in monolayers of cells: control 0 h (**a**) and 24 h (**b**), baltergin 25 µg/mL (**c**) and 50 µg/mL (**e**), baltergin-Na_2EDTA 25 µg/mL (**d**) and 50 µg/mL (**f**). **g** Quantitative analysis of cell migration. *Each bar* shows the mean ± SD of three independent experiments that were performed in triplicate. *$p < 0.05$ compared with control 0 h

performed with high molecular weight metalloproteinases from a number of Viperidae species in order to elucidate the complex integrin-disintegrin interactions. SVMPs containing disintegrin-like domains (PIII/PIIIb class) may play a role in targeting the protein to a particular site in cells such as platelets, and endothelial cells, as well as in integrins, extracellular matrix and other substrates.

Recently, a two-step model was explained by Gutierrez et al. [28], for the mechanism of action of hemorrhagic SVMPs. Briefly, in a first step SVMPs bind to and hydrolyze critical structural components of the basement membrane of capillary vessels that results in the mechanical weakening of this scaffold structure. As a consequence, in a second step, the biophysical hemodynamic forces normally operating in the microcirculation induce a distention of the vessel wall until the capillary is eventually disrupted, with the consequent extravasation of blood [28].

Baltergin is a member of P-III class of SVMPs isolated from *B. alternatus* venom which is responsible to induce relevant local effects. Previous works from our group demonstrated in vivo myotoxic effects of this metalloproteinase [3]. After intramuscular injection in murine gastrocnemium muscle, the metalloproteinase induced a late increase in serum creatine kinase, characteristic of muscle damage induced by SVMPs [29]. Also, we evaluated the capacity of this protein to induce cellular death and interestingly, this metalloproteinase showed a rapid dose-dependent detachment of myoblast (C2C12) and endothelial (t-End) cells but without cytolysis [5, 6]. In the present study, C2C12 cell line was also used. These cells are an appropriate in vitro

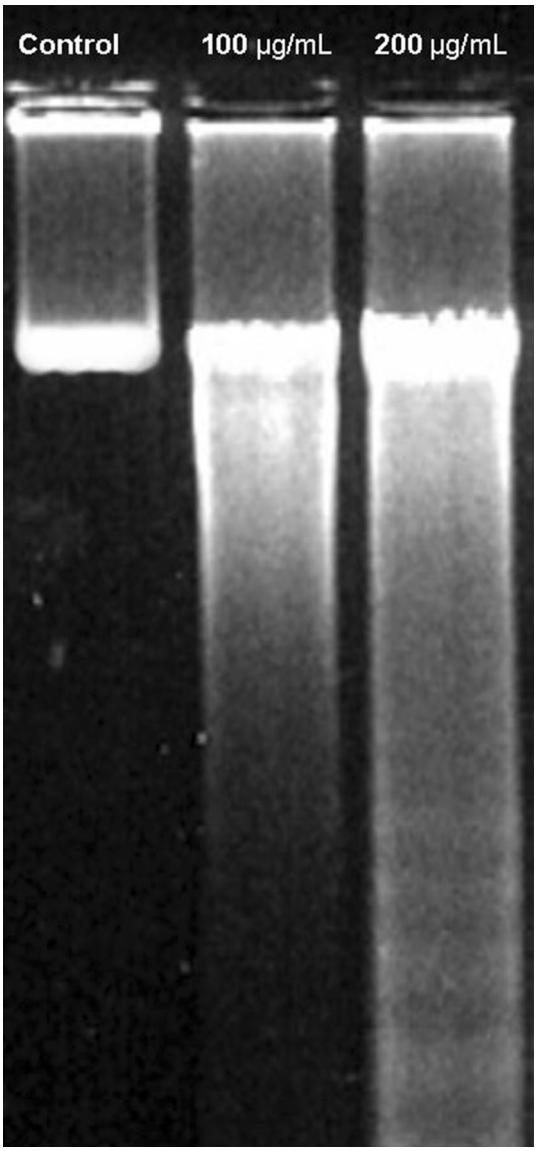

Fig. 6 Electrophoretic examination of genomic DNA of C2C12 cells. Genomic DNA was extracted and analyzed by electrophoresis on the 1.5% agarose gel containing ethidium bromide. Line 1: Control, Line 2: baltergin 100 µg/mL, Line 3: baltergin 200 µg/mL

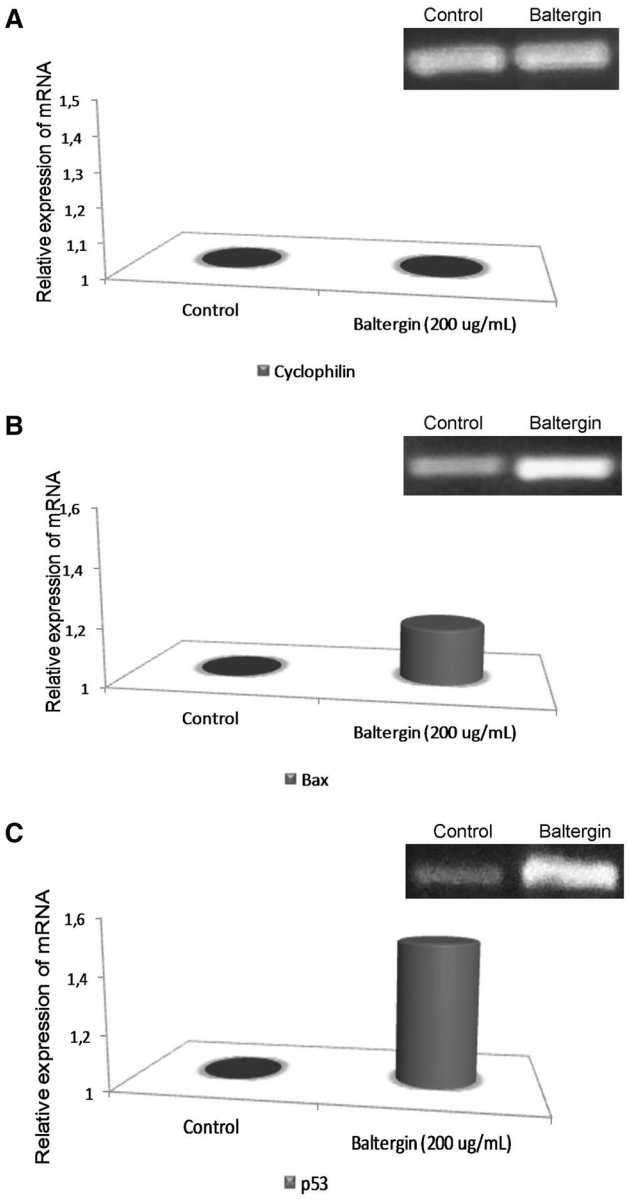

Fig. 7 Effect of baltergin (200 µg/mL) on mRNA expressions of p53 and bax in C2C12 cells. The levels of p53 and bax mRNA were detected using the reverse transcription-polymerase chain reaction (RT-PCR) analysis. Cyclophilin mRNA was used as the internal control. **a** Cyclophilin control, **b** Bax mRNA expression, **c** p53 mRNA expression

model system and a suitable target for the cytotoxic action of crude venoms or isolated enzymes, as proven by previous studies [8–11, 13, 30]. Baltergin induces apoptotic morphological changes in these myoblast cells. Under the phase-contrast microscope, baltergin-treated cells showed cell shrinkage, cytoplasm condensation, and irregularity in shape. Besides, dual acridine orange/ethidium bromide (AO/EB) fluorescent staining was used to identify apoptosis-associated changes of cell membranes. Fluorescence staining evidenced that cell membrane remains intact after toxin treatment exhibiting green but not red/orange fluorescence, indicative of acridine orange retention and ethidium bromide exclusion. Morphological changes associated with apoptosis were observed, principally, membrane blebbing and apoptotic bodies. The scanning electron microscopy also confirms these changes with the detection of cell shrinkage and once more membrane blebbing and the presence of apoptotic bodies.

On the other hand, cell adhesion is a critical event in many biological phenomena such as development, differentiation, signals transduction, maintenance of tissue

structure, wound healing, and tumor metastasis. Moreover, a vital step in the invasive cell processes is adhesion, cell migration and angiogenesis [60]. Therefore, two another approaches to this study were the evaluation of the in vitro toxin effect on cell adhesion and migration processes. The inhibition of adhesion promoted by baltergin was similar to those reported in other studies were metalloproteinases containing disintegrin-like domains, or disintegrins, inhibited cellular adhesion [31–34]. Our results demonstrated that baltergin induced a disruption of focal adhesion signaling, followed by loss of cell anchorage without involving a cytolytic effect, as indicated by the lack of release of cytosolic LDH to supernatants in cells exposed to the enzyme.

Besides, this toxin was also able to inhibit cell migration in a dose dependent manner. In this assay, the inhibition of baltergin with Na_2EDTA showed the partial role of the catalytic activity in this effect. Thus, probably, other non-catalytic mechanisms could be involved.

Integrins are cell surface receptors that play critical roles in both processes (adhesion and migration). For this reason, it is possible that these effects were due in part to the specific binding or interaction of this toxin to $\alpha\beta$ integrins and their subtypes. Previously, it has been shown that classical RGD-disintegrins induce apoptosis by interfering with αvβ3 attachment to ECM proteins [16, 35, 36] The absence of appropriate ECM contacts triggers apoptosis, and this programmed mechanism of cell death in response to lack of adhesion or inappropriate adhesion has been termed *anoikis* [15]

It is known that this mechanism of programmed cell death involves the activation of endonucleases resulting in the cleavage of genomic DNA into well-defined fragments that appear as the characteristic ladder pattern upon agarose gel electrophoresis [37]. To provide evidence supporting the involvement of apoptosis in the baltergin-induced cytotoxicity, the DNA fragmentation assay was performed and the distinctive ladder pattern characteristic of apoptotic cell death was detected.

Consistent with the induction of apoptosis, this toxin increased the expression of proapoptotic proteins. Apoptosis can be triggered through either the intrinsic and/or the extrinsic pathways. The intrinsic pathway is mediated through a mitochondria-dependent mechanism that is regulated by members of the Bcl-2 family. The p53 protein is a fundamental mediator in cellular processes of growth arrest and apoptosis and directly activates the proapoptotic Bcl-2 proteins. Our results showed that baltergin up-regulated the expression of p53 and Bax, one of the key members of the Bcl-2 family which convert from harmless monomers into deadly oligomers that form pores in the mitochondrial outer membrane [38].

Snake venom components captivate medical interest as potential molecules for the treatment of tumors [1, 16, 39–42]. The design of anti-migratory compounds is a particularly promising approach because these compounds not only delay tissue invasion and the formation of metastases by cancer cells emerging from primary tumor sites, but also restore a certain level of sensitivity to apoptosis and/or autophagy in these slowly migrating cells [43]. In this context many toxins from snake venoms have been investigated as a possible treatment for cancer; among these compounds are PLA_2s, SVMPs, disintegrins, C-type lectins, etc [30, 41, 44–49]. In particular, SVMPs are promising prototypes because of their capacity of degrading various proteins of the basement membrane, as well as cell components involved in cell-matrix and cell–cell adhesion [50], as it was demonstrated in this work for baltergin.

This is the first report that demonstrated the mechanism of programmed cell death triggered by a PIII metalloprotease isolated from *B. alternatus* venom. The present data show that baltergin causes apoptosis, specifically anoikis, of myoblast cells directly related to the detachment effect and probably due to the interaction of this toxin with integrins.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest regarding this manuscript.

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