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Blood flow is required for rapid endothelial cell damage induced by a snake venom hemorrhagic metalloproteinase

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Abstract

The effects of blood flow interruption on the ultrastructural alterations induced by a snake venom hemorrhagic metalloproteinase on skeletal muscle capillary endothelial cells were studied. Saline solution or the metalloproteinase BaP1, from the venom of *Bothrops asper*, was injected into the gastrocnemius muscles of mice with normal blood flow perfusion or with blood supply abrogated by two different protocols. Tissue was collected 5 min after injection, and both histological and ultrastructural analyses of the muscle capillary vessels were performed. Muscle with normal perfusion injected with saline solution had the typical morphology of normal capillaries, whereas injection of metalloproteinase to muscle with normal blood flow induced prominent degenerative changes in endothelial cells, such as reduction in cell thickness, decrease in the amount of pinocytotic vesicles, prominent distention and rupture leading to extravasation. In contrast, endothelial cells of capillaries from tissue devoid of blood flow and injected with the metalloproteinase did not show degenerative changes. The only alterations observed were a reduction in the capillary lumen and the presence of cytoplasmic projections, or 'pseudopods', both of which were also present in capillaries from tissue devoid of blood flow and injected with saline solution, thus suggesting that such changes are due to the drop in transmural pressure as a consequence of blood flow interruption. Our observations support the hypothesis that biophysical forces operating in the microvasculature, i.e., transmural pressure-dependent wall tension and shear stress, play a significant role in the pathogenesis of endothelial cell damage and hemorrhage induced by snake venom metalloproteinases.

Keywords: Snake venom metalloproteinases; Endothelial cells; Capillaries; Hemorrhage; Transmural pressure; Shear stress

Introduction

Envenomations by snakes of the family Viperidae are common, particularly in tropical and subtropical regions of Asia, Africa and Latin America (Warrell, 1995a,b, 2004). Such envenomations are characterized by a complex pathophysiology which includes prominent local tissue damage and diverse systemic alterations (Gutiérrez and Lomonte, 2003; Warrell, 2004). Local and systemic hemorrhages are common manifestations in victims of viperid snakebites (Cardoso et al., 1993; Otero et al., 2002; Warrell, 1995a). In these cases, bleeding plays a key role in local tissue pathology, cerebrovascular accidents and severe hemodynamic disturbances which may lead to cardiovascular shock (Warrell, 1995a; Otero et al., 2002). Zinc-dependent metalloproteinases of the 'metzincin' family of metalloproteinases have been identified as the main hemorrhagic snake venom components (Bjarnason and Fox, 1994; Fox and Serrano, 2005; Gutiérrez and Rucavado, 2000). However, despite the fact that snake venom metalloproteinases (SVMPs) have been thoroughly characterized in their biochemistry and pathological effects (Ownby, 1982; Ownby et al., 1978; Moreira et al., 1994; Fox and Serrano, 2005), the precise mechanism by which they provoke hemorrhage remains elusive.

The studies performed on the mechanism of action of hemorrhagic SVMPs have allowed the following observations: (a) SVMPs are able to degrade, in vitro, the most important components of the basement membrane, such as laminin, type IV collagen and nidogen/entactin (Ohsaka et al., 1973; Baramova et al., 1989, 1990; Rucavado et al., 1995). Although such degradation has not been directly demonstrat-

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ed in vivo, ultrastructural observations in capillary blood vessels strongly support the occurrence of such effect in envenomated tissues (Ownby and Geren, 1987; Ownby et al., 1978; Moreira et al., 1994). (b) Hemorrhage occurs predominantly in the capillary segment of the microvasculature, associated with prominent ultrastructural alterations of endothelial cells within the first minutes of SVMPs injection (Ownby and Geren, 1987; Ownby et al., 1978, 1990; Moreira et al., 1994; Anderson and Ownby, 1997). The most typical early morphological changes observed include a reduction in the thickness of cells, a drop in the number of pinocytotic vesicles and a separation of cells from their basement membrane, events that are followed by the disruption in the cellular integrity, leading to extravasation (Ownby et al., 1978; Moreira et al., 1994; Gutiérrez et al., 2005). (c) In contrast with in vivo observations, incubation of SVMPs with endothelial cells in culture does not result in rapid cell damage. Instead, the most notorious alteration is a detachment of cells from their substratum, an event not associated with loss of viability (Lomonte et al., 1994; Borkow et al., 1995; Rucavado et al., 1995; Díaz et al., 2005). At later time intervals, cells undergo apoptosis/anoikis, which probably depends on loss of contact with their matrix (Wu and Huang, 2003; You et al., 2003; Díaz et al., 2005; Tanjoni et al., 2005). Thus, SVMPs are not acutely cytotoxic to endothelial cells in vitro and yet induce prominent and rapid endothelial cell pathology in vivo.

These apparently puzzling observations have been recently put together in a unified hypothesis which incorporates the biophysical forces acting in the microvasculature in vivo (Gutiérrez et al., 2005). According to this hypothesis, SVMP-induced hemorrhage occurs by a two-step mechanism: (a) initially, SVMP degrades basement membrane components, thereby affecting its mechanical properties. This initial effect does not cause per se any major pathological alteration in endothelial cells. (b) As a consequence of the weakening of the basement membrane, endothelial cell damage occurs due to the biophysical forces which normally operate in vivo, i.e., transmural pressure and shear stress (Milnor, 1980; Ballerman et al., 1998; Schmid-Schönbein, 1999). The distention of the capillary wall and the consequent increment in wall tension induce a reduction in the thickness of endothelial cells up to a point when cells are disrupted, as has been described in ultrastructural observations (Gutiérrez et al., 2005).

This hypothesis allows several predictions that can be assessed experimentally. If biophysical forces, which depend on blood flow through the microvasculature, are indeed so relevant for endothelial cell pathology, the abrogation of such forces by elimination of blood flow would then preclude the described alterations in endothelial cells. Such experimental testing was performed in this work in which blood flow through the gastrocnemius muscle of mice was abrogated. Results clearly indicate that, under these circumstances, the injection of a hemorrhagic metalloproteinase from the venom of *Bothrops asper* does not induce endothelial cell pathology.

Materials and methods

Metalloproteinase

The SVMP BaP1 was purified from *B. asper* venom by a combination of ion-exchange chromatography on CM-Sephadex C-25 and affinity chromatography on Affi-Gel Blue, as previously described (Gutiérrez et al., 1995; Rucavado et al., 1998). It is a 23-kDa protein comprising the metalloproteinase domain only, being therefore a P-I SVMP (Watanabe et al., 2003). BaP1 induces hemorrhage, as well as other local pathological effects, upon intramuscular injection in mice (Gutiérrez et al., 1995; Rucavado et al., 1995).

Experimental protocol

The experimental protocol used was approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUA) of the University of Costa Rica. CD-1 mice (18–20 g) were anesthetized with a mixture of ketamine and xylazine. They were then divided into six groups of four mice each, which were treated as follows: *Group 1*: the left gastrocnemius muscle was exposed, and 50 μl of sterile phosphate-buffered saline solution, pH 7.2 (PBS), was injected directly into the middle portion of the muscle. *Group 2*: the muscle was exposed identically as in group 1, and 60 μg of BaP1, dissolved in 50 μl of PBS, was injected as described. *Group 3*: the left gastrocnemius muscle was exposed, and its connections were severed with a surgical blade in such a way that the muscle was completely separated from the surrounding tissues, except for its distal connection with the tendon. Then it was injected with 50 μl of PBS as described. *Group 4*: the muscle was exposed and separated from its connections, as indicated above. Then it was injected with 60 μg of BaP1, dissolved in 50 μl of PBS. *Group 5*: the left gastrocnemius muscle was exposed, and a ligature was placed proximally, just above the gastrocnemius, in order to interrupt the blood flow through the muscle. Then muscle was directly injected with 50 μl of PBS. *Group 6*: a similar procedure described for group 5 was followed. Then muscle was injected with 60 μg of BaP1, dissolved in 50 μl of PBS.

In all groups, 5 min after injection of PBS or BaP1, mice were killed by an overdose of anesthetic. The injected gastrocnemius muscles were dissected out, cut into 1-mm pieces and immediately added to Karnovsky's fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2). Postfixation was performed for 1 h in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.2. Samples were then dehydrated in serial ethanol solutions and embedded in Spurr resin. Thick (1 μm) sections were stained with toluidine blue and observed in a light microscope. Thin (gold to silver) sections were stained with uranil acetate and lead citrate and observed in a transmission electron microscope. Tissue blocks were selected at random, and the morphology of 50–60 capillaries, located in the vicinity of muscle fibers at the periphery of muscle fascicles, was observed for each experimental group. Each capillary observed was classified within the following morphological categories: (a) capillaries of normal ultrastructure; (b) capillaries with thinning of endothelial cells, drop in pinocytotic vesicles and interruptions in endothelial cell integrity; (c) capillaries with thinning or disappearance of basement membrane; and (d) capillaries with corrugated morphology, reduced lumen or having cytoplasmic projections ('pseudopods'); morphological categories (b) and (c) are not mutually exclusive, since capillaries having alterations in endothelial cells may also have damage to basement membrane. The frequency of capillaries for each of these categories was then quantified. Tissue sampling was performed 5 min after injection because previous work has shown that the onset of hemorrhage occurs within the first minutes in mouse skeletal muscle injected with SVMPs (Ownby and Geren, 1987; Ownby et al., 1978; Moreira et al., 1994). Moreover, endothelial cell ultrastructural alterations induced by ischemia take a longer time to develop. Hence, by examining tissue at 5 min, we assured that the ultrastructural changes that might be observed in tissue where blood flow was interrupted are not due to ischemia.

Distribution of BaP1 in the muscle

In order to assess if the SVMP is distributed similarly in muscle with blood flow and without blood flow, the following experiment was performed.

BaP1 was labeled with fluorescein (FluoReporter FITC protein labeling, Molecular Probes), following the instructions of the manufacturer. However, since BaP1 has a molecular mass of 23 kDa, a 10-kDa cut-off centrifugal filter (Microcon, Amicon, Bedford, MA) was used instead of the spin column provided by the manufacturer (30 kDa cut-off), in order to separate free fluorescein from protein-bound fluorescein. The recovery of BaP1 after removal of free fluorescein was demonstrated by SDS-PAGE, using 15% gels, run under reducing conditions (Laemmli, 1970). Mice were anesthetized as described, and the left gastrocnemius muscle was exposed. Three mice were injected directly in the middle portion of the muscle with 60 μg of fluorescein-labeled BaP1, in a volume of 50 μl. In another group of three mice, the connections of gastrocnemius muscle were severed with a surgical blade, in such a way that the muscle was completely separated from the surrounding tissues, except for its distal connection with the tendon. Then the same dose of fluorescein-labeled BaP1 was injected as described above. Five minutes after BaP1 injection, mice of the two groups were killed by an overdose of anesthesia, and the gastrocnemius muscles were immediately frozen in dry ice-cold isopentane. Five-micrometer cross-sections were immediately prepared in a cryostat (Leica CM 1850, Nussloch, Germany) and observed in a fluorescence microscope.

Results

Histological observations

The histological and ultrastructural characteristics of tissue samples of the four mice included within each experimental group were very similar, and no intragroup differences were observed. Therefore, the description of these alterations will be performed for each group.

Group 1

Muscle tissue had the characteristic features of normal skeletal muscle. The integrity of muscle fibers was conserved, the interstitial space area had typical characteristics, and there was no evidence of hemorrhage or inflammation, as leukocytes were absent in the endomysium and perimysium. Capillary vessels were normal, and some of them contained erythrocytes in their lumen (results not shown).

Group 2

Prominent hemorrhage was observed in areas of the tissue, evidenced by groups of extravasated erythrocytes in the endomysium and perimysium (Fig. 1A). Skeletal muscle fibers did not show evidence of myonecrosis, and the area of interstitial space was increased when compared to group 1, probably due to extravasation and edema. Intact capillary vessels were largely absent in regions of hemorrhage located predominantly at the peripheral areas of muscle fascicles.

Groups 3, 4, 5 and 6

Tissue from these experimental groups showed the same histological features, characterized by the lack of hemorrhage, i.e., absence of extravasated erythrocytes. No inflammatory cells were present in the tissue, and skeletal muscle fibers did not present evidences of cellular damage. Abundant microvessels, i.e. capillaries and venules, of normal appearance were observed (Fig. 1B).

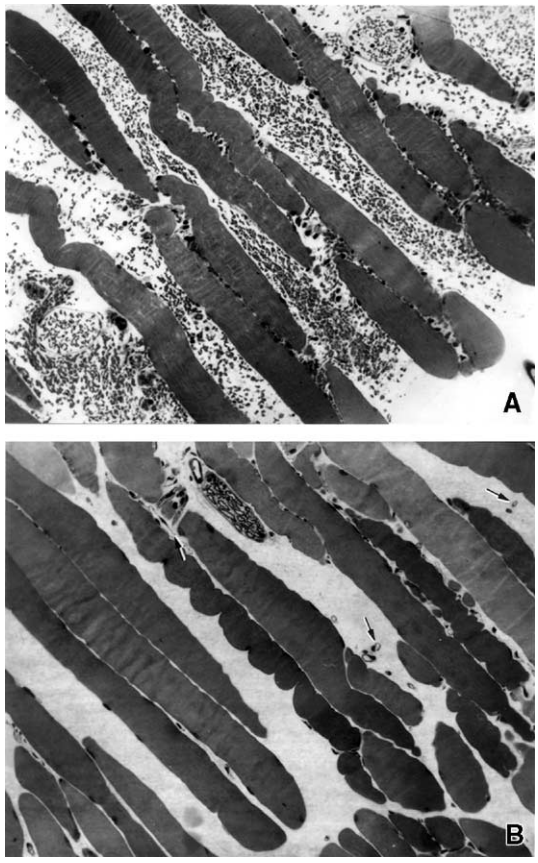

Fig. 1. Light micrographs of sections of mouse gastrocnemius muscle 5 min after injection of 60 μg of metalloproteinase BaP1 in conditions of normal blood flow (experimental group 2) (A) and absence of blood flow due to surgical separation of the muscle from its vascular supply (experimental group 4) (B). Notice the absence of hemorrhage in panel B, with microvessels showing normal morphology (arrows), whereas prominent hemorrhage and very few intact capillaries are observed in panel A. Toluidine blue stain. 170×.

Ultrastructural observations

Group 1

Capillary vessels presented an array of normal morphological features, such as abundant pinocytotic vesicles, intact intercellular junctions and a continuous basement membrane surrounding endothelial cells (Fig. 2). There were no signs of hemorrhage, as no erythrocytes were present in the interstitial space. The morphology of nuclei and mitochondria in endothelial cells were also normal (Table 1).

Group 2

Twenty-nine out of 53 capillaries examined showed marked morphological alterations, whereas 24 capillaries did not present these degenerative changes (Table 1). In general, affected capillaries were observed in regions of hemorrhage, i.e., areas with extravasated erythrocytes. Affected endothelial cells had a drastic reduction in the number of pinocytotic vesicles which, in some cells, were completely absent. The thickness of endothelial cells was greatly reduced, and cells were overly distended, with an increased lumen (Figs. 3 and 4). A conspicuous localized distention of the capillary wall was

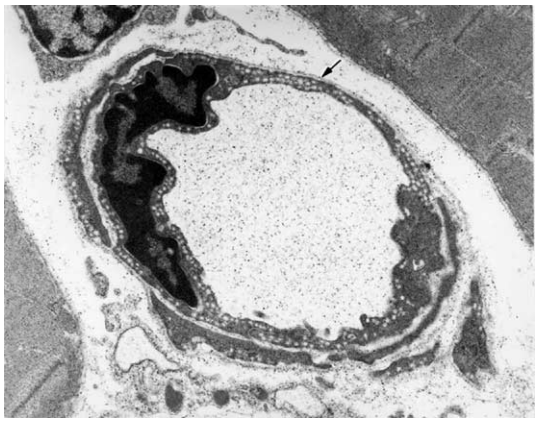

Fig. 2. Electron micrograph of a capillary vessel from the gastrocnemius muscle of mouse injected with PBS and having a normal blood flow (experimental group 1). Endothelial cell has a normal ultrastructure, with abundant pinocytotic vesicles and an intact basement membrane (arrow). 17,000×.

observed in some points, with the distended cell having a very thin structure (Fig. 4). In addition, the integrity of endothelial cells was interrupted at some points in affected capillaries (Fig. 3), and the basal lamina had a reduced thickness or was absent (Figs. 3 and 4). Abundant erythrocytes were observed in the endomysium in areas where capillaries showed pathological alterations. These changes are very similar to the ones previously described as a consequence of the action of purified SVMPs (Ownby and Geren, 1987; Ownby et al., 1978; Moreira et al., 1994).

Groups 3, 4, 5 and 6

Similar ultrastructural features were observed in endothelial cells in these four experimental groups; therefore, a single qualitative description will be performed for all of them; a quantitative assessment of the frequency of the morphological features observed is presented in Table 1. Endothelial cells did not present ultrastructural evidences of degenerative changes, such as those described for group 2; there was not a single case of endothelial cell damage in any of the sections examined (Table 1). However, the lumen of many capillary vessels was notoriously reduced, and endothelial cells had a corrugated appearance (Figs. 5–7). Cells had a high number of pinocytotic vesicles, and the thickness of the cells was apparently larger than that of endothelial cells injected with either PBS or BaP1 and submitted to normal perfusion (groups 1 and 2). The majority of endothelial cells examined had cytoplasmic projections (Figs. 5–7; Table 1) similar to the 'pseudopods' described by Lee and Schmid-Schönbein (1995) in capillaries in conditions where transmural pressure was brought to 0 cm H_2O. There were no evident alterations in the thickness of the basement membrane of all capillaries examined in these four experimental groups (Figs. 5–7; Table 1). No evident ultrastructural differences were observed in endothelial cells of capillaries from muscle devoid of blood flow and injected with either PBS or SVMP BaP1 (Table 1).

Distribution of BaP1 in the muscle

The distribution of fluorescein-labeled BaP1 5 min after injection in gastrocnemius muscle was similar either in the presence or absence of blood flow. In both experimental groups, fluorescence was predominantly observed in the perimysium surrounding muscle fascicles, with penetration into the endomysium of several layers of peripheral muscle fibers (Fig. 8).

Discussion

Previous investigations revealed a puzzling difference on the action of hemorrhagic SVMPs in vivo and in endothelial cell culture, since prominent alterations occur in the former conditions, whereas only detachment is described in the latter (see Gutiérrez et al. (2005) for a review). Our results demonstrate that capillary vessel damage associated with prominent endothelial cell distention and rupture, which develops rapidly after intramuscular injection of the hemorrhagic SVMP BaP1, occurs only in conditions of blood flow. Interruption of blood flow, by two different experimental procedures, results in a complete absence of capillary endothelial cell degeneration in the tissue injected with BaP1.

Table 1
Quantitative assessment of the frequency of various ultrastructural alterations observed in capillaries from muscle tissue of animals submitted to the experimental protocols used in this study

Experimental group[a]	Total number of capillaries examined	Capillaries with normal structure[b]	Capillaries with thinning of endothelial cells, drop in pinocytotic vesicles and interruptions in endothelial cell integrity	Capillaries with thinning or disappearance of basement membrane	Capillaries with corrugated morphology, reduced lumen or having cytoplasmic projections ('pseudopods')
1	56	56	0	0	0
2	53	24	29	29	0
3	50	4	0	0	46
4	58	8	0	0	50
5	52	7	0	0	45
6	55	6	0	0	49

[a] For the details of each experimental group, see the Materials and methods section.
[b] Refers to the morphology observed in capillaries from tissue with normal blood flow injected with PBS and corresponds to the characteristic ultrastructural features described in previous studies for normal skeletal muscle capillary vessels (see the Results section for details).

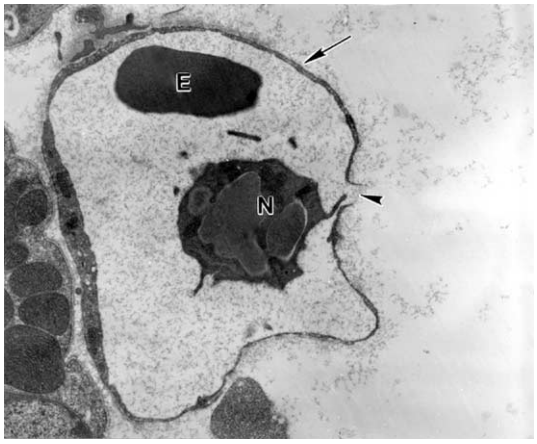

Fig. 3. Electron micrograph of a capillary vessel from the gastrocnemius muscle of mouse injected with 60 μg of metalloproteinase BaP1 under normal blood flow (experimental group 2). The endothelial cell is distended and has a reduced thickness, being almost devoid of pinocytotic vesicles. A rupture in endothelial cell is observed (arrowhead), and the basement membrane shows disorganization and loss of structure (arrow). A neutrophil (N) and an erythrocyte (E) are present in the capillary lumen. 10,000×.

SVMPs are known to readily cleave basement membrane components in vitro (Ohsaka et al., 1973; Baramova et al., 1989, 1990; Rucavado et al., 1995), and such cleavage is also likely to occur in vivo, although this has not been properly demonstrated. On the other hand, the effect of SVMPs on endothelial cells largely depends on the context in which these cells are located. Injection of hemorrhagic SVMPs in vivo results in rapid and drastic pathological alterations in capillary endothelial cells, associated with an early onset of hemorrhage, as described in ultrastructural (Ownby and Geren, 1987; Ownby et al., 1978; Moreira et al., 1994; Anderson and Ownby, 1997) and intravital microscopic (Rucavado et al., 1995) observations. However, when SVMPs are incubated with various types of endothelial cells in culture, no rapid

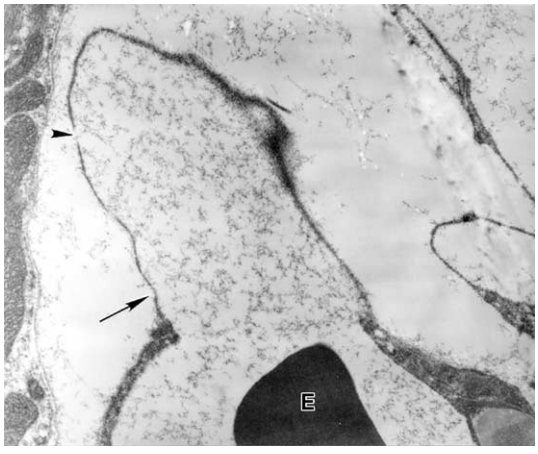

Fig. 4. Electron micrograph of a portion of a capillary vessel from the gastrocnemius muscle of mouse injected with 60 μg of metalloproteinase BaP1 under normal blood flow (experimental group 2). Endothelial cell shows a drastic distention in one segment (arrow) in which the cell has become extremely thin, with its integrity interrupted at some points (arrowhead). Notice the absence of basement membrane. A portion of an erythrocyte (E) is present in the lumen. 22,000×.

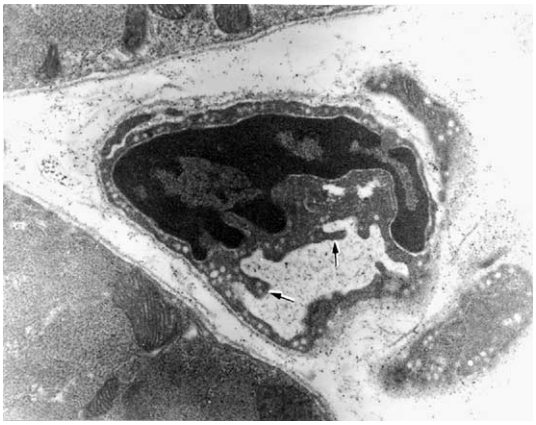

Fig. 5. Electron micrograph of a capillary vessel from the gastrocnemius muscle of mouse injected with PBS but lacking blood flow due to separation of muscle from its vascular supply (experimental group 3). Endothelial cell has a normal morphology, but there is a reduction in the lumen of the vessel. In addition, there are cytoplasmic projections, or 'pseudopods', protruding to the lumen (arrows). 18,000×.

cytopathological effects occur. The most notorious alteration in these conditions is the detachment of cells from their substratum, without loosing their viability within the first hours (Lomonte et al., 1994; Borkow et al., 1995; Rucavado et al., 1995). Later on, they undergo apoptosis/anoikis, probably as a consequence of the separation from the matrix (Díaz et al., 2005; Tanjoni et al., 2005).

An explanation for these apparently contradictory findings has been recently presented in a hypothesis that proposes a key role for the biophysical forces which operate under normal hemodynamic conditions in the microvasculature (Gutiérrez et al., 2005). According to such hypothesis, capillary damage in vivo occurs through a 'two-step' mechanism. The first step is based on a proteolytic degradation of basement membrane components, in such a way that the mechanical stability of this extracellular matrix scaffold becomes significantly impaired. The Young's modulus, i.e., the force per unit area required to extend the material 100% (Milnor, 1980), of the capillary wall depends largely on the mechanical properties of the basement membrane (Murphy and Johnson, 1975; Lee and Schmid-Schönbein, 1995), although endothelial cell cytoskeleton also plays a mechanical role (Gottlieb et al., 1991; Lee and Schmid-Schönbein, 1995). Hence, the weakening of the basement membrane, through proteolysis of mechanically relevant peptide bonds of its constituent proteins, would reduce its Young's modulus and, therefore, would greatly increment the distensibility of the capillary wall. As a consequence, under normal transmural pressures, this brings up a distention of the capillary wall, with an increment in wall tension, leading eventually to endothelial cell damage and capillary wall disruption (Gutiérrez et al., 2005). Another biophysical force that might contribute to endothelial cell damage is the shear stress, which depends on blood flow, blood viscosity and the diameter of the microvessel (Ballerman et al., 1998; Schmid-Schönbein, 1999). It is suggested that shear stress is likely to affect endothelial cells whose attachment with the basement membrane has been impaired as a consequence of SVMP-

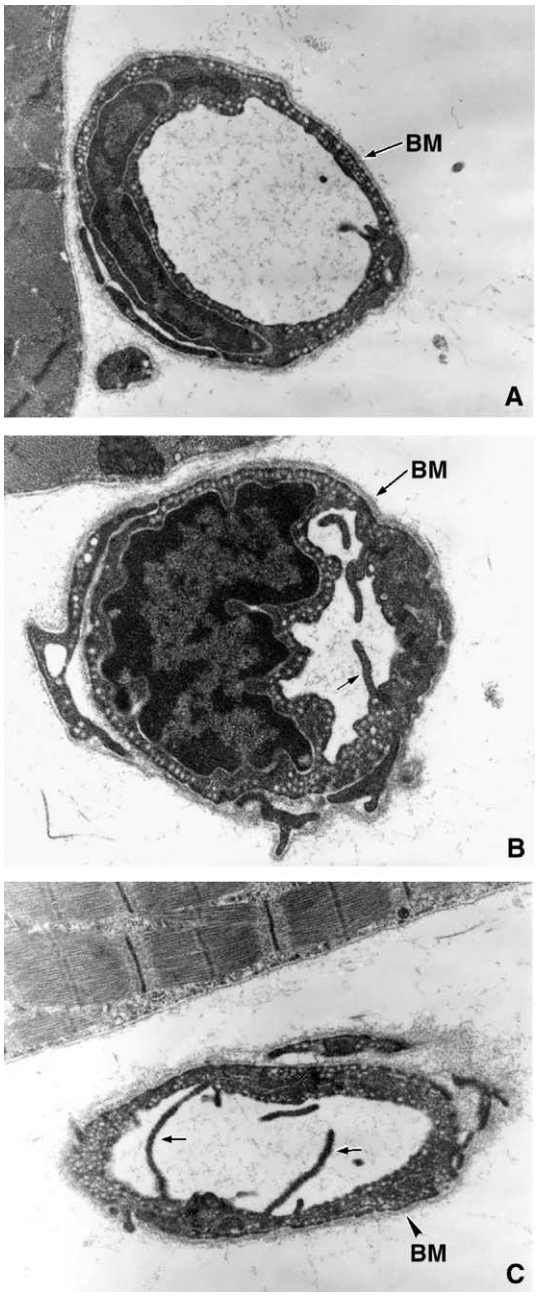

Fig. 6. Electron micrographs of capillary vessels from the gastrocnemius muscle of mouse injected with 60 μg of metalloproteinase BaP1 under conditions of lack of blood flow due to surgical separation of the muscle from its vascular supply (experimental group 4). Endothelial cells do not show signs of degeneration and present abundant pinocytotic vesicles. The basement membrane (BM) has normal ultrastructural features. In panel B, notice a reduction in the lumen of the capillary. Cytoplasmic projections, or 'pseudopods', are abundant in endothelial cells in panels B and C (arrows). (A) 18,000×, (B) 20,000×, (C) 18,000×.

induced proteolysis (Gutiérrez et al., 2005), as has been observed in capillaries affected by some SVMPs (Moreira et al., 1994).

The ultrastructural alterations observed in samples from group 2 of our work, i.e., in mice injected with BaP1 under normal blood flow conditions, and in previous studies on the pathogenesis of hemorrhage (Ownby et al., 1978; Moreira et

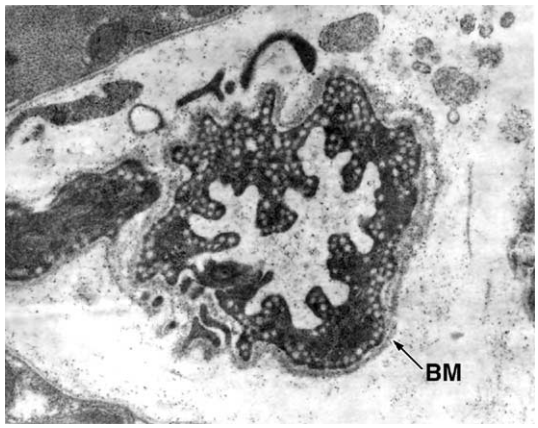

Fig. 7. Electron micrograph of a capillary vessel from the gastrocnemius muscle of mouse injected with 60 μg of metalloproteinase BaP1 under conditions of suspension of blood flow due to the application of a ligature (experimental group 6). No evidence of endothelial cell damage is observed, and abundant pinocytotic vesicles are present. There is a reduction in the capillary lumen, and the cell has a corrugated appearance, with the presence of cytoplasmic projections. The basement membrane (BM) is present around the capillary and does not show evident alterations. 17,000×.

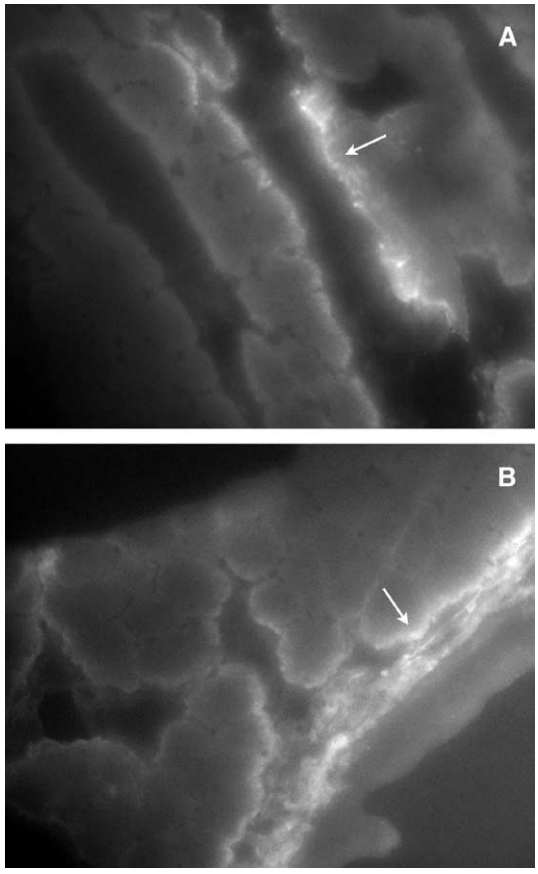

Fig. 8. Fluorescence micrograph of mouse gastrocnemius muscle injected with fluorescein-labeled metalloproteinase BaP1 under conditions of normal blood flow (A) or interrupted blood flow (B). A similar distribution of BaP1 is observed in the perimysium surrounding muscle fascicles and in the endomysium within the most peripheral layers of muscle fibers. Arrows show areas of fluorescence. 70×.

al., 1994), are compatible with this hypothesis. The capillary wall is distended, and the thickness of endothelial cells is reduced as a consequence of SVMP action. Moreover, the number of pinocytotic vesicles is greatly diminished. These vesicles represent a reserve of membrane that can be translocated to the plasma membrane by exocytosis, thus increasing the surface area of the membrane in conditions of distention, as has been demonstrated experimentally under increased transmural pressure (Lee and Schmid-Schönbein, 1995). In contrast, under zero pressure, the number and membrane area of these vesicles increase (Lee and Schmid-Schönbein, 1995). Therefore, when endothelial cells are distended due to increased transmural pressure or, as might occur in our case, to an increment in the distensibility of capillary wall due to a proteolysis-mediated weakening of the basement membrane, the number of pinocytotic vesicles is reduced. Eventually, this adaptation of endothelial cells to distention is likely to be overcome and cells are disrupted, with the consequent extravasation.

When blood flow was interrupted, no endothelial cell pathology was observed in any of the capillaries examined. The only evident ultrastructural alteration was a reduction in the lumen of capillaries and the acquisition of a corrugated appearance of these cells with abundant pinocytotic vesicles. However, no morphological signs of cell degeneration were observed in any of the capillaries studied, as the ultrastructure of the organelles was normal. Thus, a direct effect of BaP1 on endothelial cell within the first minutes of injection is highly unlikely, in agreement with results obtained in cell culture conditions with this and other SVMPs (Lomonte et al., 1994; Borkow et al., 1995; Rucavado et al., 1995), where biophysical forces operating in vivo are absent. The reduction in capillary lumen and the morphological features of endothelial cells under no flow conditions are very similar to those described for normal rat skeletal muscle capillaries under a transmural pressure of 0 cm H_20 (Lee and Schmid-Schönbein, 1995). Another coincidence between our findings and those of Lee and Schmid-Schönbein (1995) is the appearance of cytoplasmic projections, denominated 'pseudopods' by these authors, in conditions of no flow. These structures are devoid of vesicles and seem to consist of polymerized cytoskeletal proteins (Lee and Schmid-Schönbein, 1995). Hence, the ultrastructural characteristics of endothelial cells under no flow conditions depend on the drastic reduction of transmural pressure and not on the effect of BaP1 on these cells. This is supported by the finding that identical changes occurred in endothelial cells of muscle with no blood flow injected with PBS instead of BaP1.

A potential drawback of our experimental design is that blood flow may affect the distribution of BaP1 in the muscle tissue. However, observations on the distribution of fluorescein-labeled BaP1 upon direct injection in the gastrocnemius muscle revealed a similar pattern in conditions of flow and no flow. In both cases, the SVMP was predominantly distributed in the perimysium that surrounds muscle fascicles and in the endomysium within the most peripheral layers of muscle fibers. Hence, the different pathological outcomes observed do not depend on a different access of BaP1 to capillary vessels. This further suggests that, in our experimental conditions, BaP1 reaches capillary vessels from the interstitial space and not through the bloodstream. Therefore, the presence or absence of blood flow would not greatly affect the interaction of this SVMP with the capillary basement membrane. On the other hand, the effects that a sudden interruption in blood flow exerts on endothelial cells have to be considered. Loss of normal shear stress in microvessels is associated with rapid cellular changes such as depolarization, generation of H_2O_2 and increased cytosolic Ca^{2+} (Song et al., 2001; Fisher et al., 2001). However, although these alterations may modify the susceptibility of endothelial cells to the action of SVMPs, it seems unlikely that such subtle intracellular changes would make these cells completely resistant to the action of SVMPs.

The ultrastructure of the basement membrane was apparently not affected in capillaries of tissues injected with BaP1 under conditions of no blood flow, whereas it was clearly altered in muscle with intact blood supply injected with this hemorrhagic SVMP. According to our model (Gutiérrez et al., 2005), hydrolysis of basement membrane components occurs regardless of the presence or absence of blood flow, since the toxin is able to reach this extracellular matrix structure from the interstitial space. It is suggested that BaP1 exerts, within the first 5 min of injection, a limited hydrolysis of basement membrane components in such a way that does not alter its general ultrastructural appearance, but drastically affects its mechanical stability. Thus, under no flow conditions, this effect does not result in ultrastructural alterations of this extracellular matrix structure. In conditions of blood flow, in contrast, capillary wall distention results in a conspicuous reduction in the thickness of basement membrane followed by its disruption, as observed in our experiments. In support of this, the ultrastructural appearance of basement membrane has been described to change depending on the extent of distention when different transmural pressures are applied to normal microvessels. The thickness of basement membranes is reduced as endothelial cells are stretched by increasing hydrostatic pressure (Lee and Schmid-Schönbein, 1995). Thus, as in the case of endothelial cell damage, the ultrastructural alterations observed in basement membranes after BaP1 injection in conditions of normal blood flow also seem to depend on the distention of the capillary wall in addition to the proteolytic cleavage of basement membrane proteins. It remains to be studied whether SVMPs are able to induce ultrastructural damage to basement membrane under no flow conditions at more prolonged time intervals, an issue that was beyond the objectives of this work.

In conclusion, our results indicate that a hemorrhagic SVMP does not affect the ultrastructure of capillary endothelial cells in mouse muscle tissue when blood flow is interrupted, whereas it drastically affects these cells in conditions of normal blood flow. These findings support the hypothesis that SVMP-induced damage to capillary endothelial cells occurs only after the biophysical forces associated with blood flow cause distention of capillaries weakened by the action of SVMPs on basement membranes.

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