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A paradoxical pro-apoptotic effect of thrombin on smooth muscle cells

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Abstract

Whereas thrombin (below 10 nM) is a potent mitogen, recent studies report that exposure to higher doses of thrombin could lead to apoptosis of neurons and tumor cells. Our results show that prolonged exposure (≥24 h) to thrombin (50–100 nM) exerts a pro-apoptotic effect on cultured vascular smooth muscle cells (VSMCs). This phenomenon depends on thrombin serine-protease activity but is independent of PAR-1 and -4 activation and subsequent signaling. The parallel occurrence of cell retraction and cleavage of fibronectin suggests that thrombin-induced apoptosis is consecutive to pericellular proteolysis. These data point to a new potential action of thrombin in the cardiovascular system.

Keywords: Vascular smooth muscle cells; Apoptosis; Thrombin; PAR; Fibronectin

Introduction

In several cell types, such as neurons, astrocytes [1,2], or tumor cells [3,4], thrombin has been shown to exert a dual effect, inducing cell proliferation at low concentrations and apoptosis at high concentrations, both effects being mediated by the G-protein-coupled protease-activated receptor (PAR-1). Thrombin is also a mitogen for vascular smooth muscle cells (VSMCs) at concentrations below 10 nM [5]. However, higher concentrations of thrombin may be generated locally in the vascular wall in pathological conditions, such as aneurysms or atherosclerosis [6,7], and apoptotic VSMCs may also promote thrombin formation [8]. In the present study, we show that high concentrations of thrombin result in VSMC apoptosis via a PAR-1/4-independent process. Since the proteolytic activity of thrombin is not limited to cellular receptors or plasma proteins, but extends to extracellular proteins such as fibronectin [9], thrombospondin [10], laminin [11], osteopontin [12], or matrix metalloproteinases (MMPs) [13], we examined whether thrombin could induce pericellular proteolysis leading to VSMC apoptosis.

Materials and methods

Purified proteins and reagents

Human α-thrombin was purified as previously described [14]. D-Phe-Pro-Arg-chloromethyl ketone dihydrochloride (PPACK-HCl; Calbiochem Novabiochem Corp, La Jolla, USA) was used to inhibit the active site of human thrombin (PPACK-thrombin); no remaining free PPACK was detectable in solution after the reaction. Hirudin was from Serbio (France) and C-terminal fragment of hirudin (54–65) from SIGMA. Recombinant rat PN-1 (a generous gift from Dr. Monard Friedrich Micher Institute, Basel, CH) was produced in yeast as previously described [15]. Human PAR-1 agonist SFLLRN was from Neosystem (Strasbourg, France). Human PAR-4 activating

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peptide GYPGQV [16] and amastatin [17] were from Bachem (Voisins-le-Bretonneux, France) and AYPGKF from Byosyntan. Broad spectrum matrix metalloproteinases inhibitor GM6001 and PD98059 were obtained from Calbiochem-VWR (Fontenay Sous Bois, France). Antibodies were as follows: polyclonal rabbit anti-rat fibronectin (Biogenesis, Poole, UK) and anti-ERKs (Promega, Madison, WI).

Animals

Male Lewis rats (120–130 g, IFFA CREDO, Lyon, France) were used for this study. The procedures used for the care and euthanasia of the animals were in accordance with the European Community Standards (Ministère de l'Agriculture, France; authorization No. 75–214).

Cell culture

Rat vascular (aortic) smooth muscle cells (VSMCs) were isolated from 180 to 200 g male Lewis rats and cultured in Dulbecco's minimum essential medium (DMEM, Life Technologies) with 10% fetal calf serum, as previously described [18], and cells were used for experimentation at passages 3–5. Human VSMCs were isolated using the same technique, from healthy portions of saphenous veins obtained from patients undergoing vein removal (Clinique du Parc Monceau, in accordance with local Ethical Committee, following the Institutional guidelines). Human VSMCs were cultured in smooth muscle cell basal medium 2 (Promocell, Heidelberg). VSMCs were identified by their characteristic "hill and valley" growth appearance and by immunostaining for SMC α-actin. When VSMCs reached confluence, the culture medium was replaced by serum-free medium for 1 day before treatment with human α-thrombin or other agonists in DMEM containing 0.1% (weight/volume) bovine serum albumin.

TUNEL assay

Terminal transferase dUTP nick end labeling (TUNEL) was used to visualize DNA fragmentation, according to the manufacturer's instructions (Roche Molecular Biochemicals). Cells were fixed in 3.7% paraformaldehyde for 30 min at room temperature and then permeabilized in 0.1% Triton-X-100, 0.1% sodium citrate for 2 min on ice. Slides were washed with PBS and incubated with TUNEL reaction mixture for 1 h at 37°C. After washing, the nuclei were counterstained with 10 ng/ml DAPI (4′, 6′-diamino-2-phenylindole-hydrochloride, SIGMA). The slides were mounted with Fluoprep (DAKO) and observed under an epifluorescence microscope. A positive control (1 μg/ml DNAse I treatment for 10 min after permeabilization) and a negative control (without terminal transferase) were included in each set of experiments.

Measurement of DNA fragmentation

Histone-associated DNA fragments were quantified by using a photometric enzyme immunoassay (Cell Death Detection ELISA ^{PLUS}, ROCHE) [19], following the manufacturer's procedure.

Western blot analysis

Extracellular signal-regulated kinases-1 and -2 (ERK1/2) phosphorylation was assessed by immunoblotting as previously described [20]. Briefly, VSMCs were lysed in RIPA buffer (50 mM Tris–HCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton-X100, 150 mM NaCl, pH 7.4) containing an antiprotease-cocktail (SIGMA) supplemented with 100 μM phenylarsine oxide, 50 mM NaF, and 200 mM Na_3VO_4. After SDS-PAGE and electrotransfer of 10 μg total proteins, the membranes were incubated with an antibody against phosphorylated ERKs (1:10,000) and then with an appropriate secondary antibody conjugated with HRP allowing detection by chemiluminescence. Membranes were stripped using Interchim Western Blot Recycling Kit, and reprobed with an anti-ERKs (1:20,000) following the same procedure.

Immunoblotting analysis of VSMC conditioned media for detection of fibronectin degradation was done as previously described [21].

Intracellular calcium mobilization, performed as previously described [22]

Briefly, VSMCs were loaded for 1 h at room temperature with 5 μM Fluo3-AM and 0.02% pluronic acid in DMEM, then washed with Fluo3-AM-free Tyrode solution. Confocal images were acquired using a Zeiss LSM-510 inverted confocal microscope (Carl Zeiss) with a Zeiss LD Achroplan ×40 objective (numeric aperture: 0.6). Fluo3-AM was excited by the 488-nm line of an argon laser, and emitted fluorescence was collected through a long pass 505-nm filter. Zeiss confocal software Windows NT controlled the scanner module and performed image analysis.

Immunocytochemistry

Alpha actin detection was performed on control and thrombin-treated rat VSMCs (100 nM for 72 h) previously cultured onto Labtech 8-well slides. VSMCs were fixed for 5 min with cold methanol and 10 min with 3.7% paraformaldehyde. Both primary and secondary antibodies (respectively, mouse anti-α-actin from DAKO and goat anti-mouse IgG conjugated to Alexa 488 fluor from Molecular Probes) were diluted to 100 μg/ml in PBS containing 1% bovine serum albumin. Saturation and washing steps were also performed in this buffer. The slides were mounted with Fluoprep (DAKO) and observed by confocal microscopy as described above.

Quantification of soluble TGF-β1

TGF-β1 concentration in the VSMC conditioned media was determined using a commercial ELISA kit (Promega), following the instruction of the manufacturer.

Determination of gelatinolytic activity of matrix metalloproteinase 2

Matrix metalloproteinase-2 (MMP-2) activity was quantified as previously described [23]. Briefly, samples were separated by SDS-PAGE containing 1 mg/ml gelatin as a substrate for MMP-2, under nonreducing conditions. The gel was renatured, incubated in an appropriate buffer, and stained with Coomassie blue.

Statistical analysis

Statistics were performed using a Statview 5.0 software. Results are expressed as mean ± SD unless otherwise stated. Comparisons used one-way analysis of variance with Scheffe's F test, or Wilcoxon signed ranks, as appropriate. Statistical significance was set at $P < 0.05$.

Results and discussion

Thrombin-induced apoptosis of VSMCs

Cell retraction and clustering, nuclear condensation shown by DAPI staining (Figs. 1A2 vs. A1), and DNA fragmentation (Fig. 1A5) were observed after incubation of rat aortic VSMCs with thrombin (100 nM). This effect was specific for thrombin, since it was reversed by the specific thrombin inhibitor, hirudin (Figs. 1A3, A6), and was both time-dependent (from 24 to 72 h, no effect being observed before 24 h; Fig. 1B) and dose-dependent (between 50 and 100 nM, with a 1.7-fold increase between these two concentrations; $n = 9$; $P < 0.02$). No apoptosis was detectable for thrombin concentrations lower than 50 nM in contrast to what was observed by Zain et al. [3] and Ahmad et al. [4] on tumor cells type. The apoptotic effect of thrombin depended on its action as a serine protease, since it was not mimicked by PPACK-thrombin, which lacks enzymatic activity, and was abolished in the presence of protease-nexin-1 (PN-1), a thrombin-inhibiting serpin (Fig. 1C). To determine whether the effect of thrombin was species-specific or dependent on the vascular territory, we incubated human VSMCs from

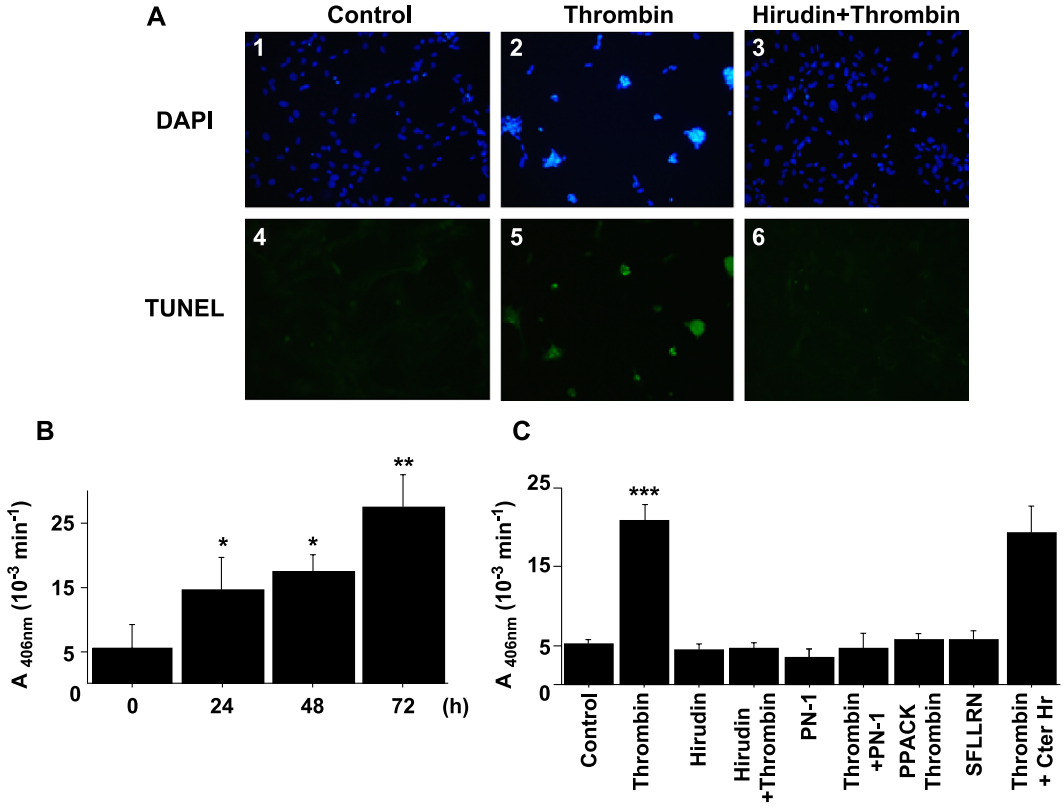

Fig. 1. Thrombin exerts a pro-apoptotic effect on rat VSMCs. (A) DAPI staining of nuclei (A1–3) and TUNEL assay (A4–6) of control cells (A1, 4), cells treated with 100 nM thrombin for 72 h without (A2, 5) or with (A3, 6) hirudin (1 μM). (B–C) Quantification of the apoptosis level induced by incubation of 100 nM thrombin for 24–72 h (B), with or without inhibitors or agonist (C) for 72 h, by the quantification of DNA fragmentation, using an ELISA as described in Materials and methods. Results are expressed in A_{405nm} 10^{-3} min^{-1} (mean ± SD, B: *$P < 0.01$ vs. control: **$P < 0.001$ vs. control C: ***$P < 0.0001$ comparing thrombin treatment with all others except thrombin + Cter hirudin) and are representative of two experiments performed independently in triplicate wells. Absorbance is directly proportional to the amount of fragmented nucleosomes.

saphenous veins with thrombin. DNA fragmentation was also induced by thrombin (100 nM) but only after a 96-h incubation (Fig. 2A).

Protease-activated receptor activating peptides do not induce VSMC apoptosis

VSMCs express the G-protein-coupled receptor PAR-1. The human PAR-1-AP, SFFLRN, used at concentrations up to 250 μM in the presence of amastatine to avoid its degradation, did not mimic the pro-apoptotic effect of thrombin either on rat or human VSMCs (Figs. 1C and 2A). Similarly, Ahmad et al. [4] could not reproduce thrombin-induced apoptosis of some tumor cells by incubation with a PAR-1 agonist. VSMCs may also express PAR-4 [24], the activation of which is not blocked by the C-terminal peptide of hirudin. As the pro-apoptotic effect of thrombin was not inhibited by hirudin C-terminal peptide, we investigated whether PAR-4 may be responsible for this effect. For this purpose, we used a potent modified mouse PAR-4 AP, AYPGKF (not shown), and the human PAR-4 AP, GYPGQV (Fig. 2A) [25]. None of these peptides was able to induce apoptosis of either rat or human VSMCs.

Apoptosis is not mediated by PAR-coupled signals

Since the aforementioned results suggest that apoptosis may occur independently of PAR activation, we investigated whether PAR-coupled responses were correctly triggered by thrombin and protease-activated receptor activating peptides (PAR-APs) in our conditions. SFLLRN, as thrombin (not shown), triggered a rapid increase in intracellular calcium concentration (as early as 3 min after stimulation, Fig. 2B), indicating that PAR-1-dependent signaling was functional. The rapid kinetics of thrombin-

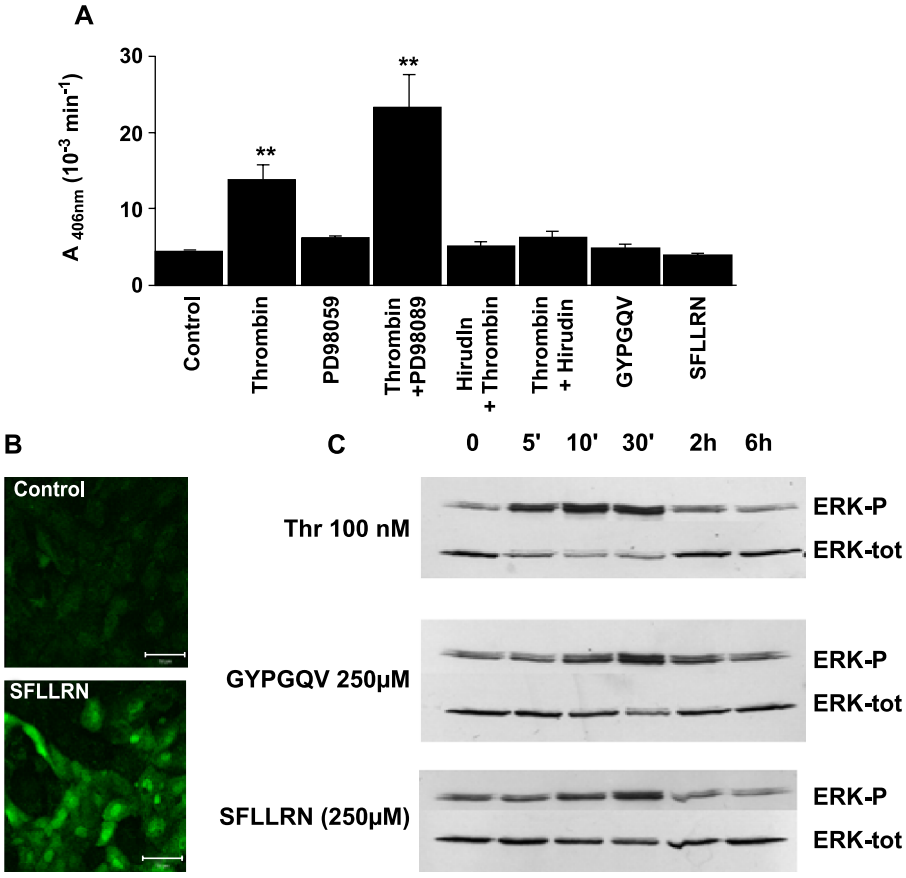

Fig. 2. Thrombin-induced apoptosis: a PAR-independent phenomenon. (A) Quantification of DNA fragmentation in human saphenous SMCs (ELISA) after 96 h of incubation. Results are expressed in A_{405nm} 10^{-3} min^{-1} (mean ± SD, $^{**}P < 0.01$ vs. control comparing thrombin treatment with all others except thrombin + PD98059, an inhibitor of ERK activation) and are representative of two experiments (primary cultures from two different patients) performed independently in triplicate wells. Hirudin + thrombin indicates that hirudin was pre-incubated for 15 min before addition of thrombin. Thrombin + hirudin indicates that hirudin was added 2 h after incubation with thrombin. GYPGQV and SFLLRN were used at 250 μM, PD98059 at 10 μM, and hirudin at 1 μM. (B) Rat aortic-cultured VSMCs express functional PAR-1, as assessed by the visualization of intracellular calcium mobilization after the addition of SFLLRN (250 μM)(as described in Materials and methods). The photomicrographs presented here were taken before (control) and 180 s after SFLLRN addition. (C) Kinetics of ERK-1/2 activation on human saphenous VSMCs in response to 100 nM thrombin, and 250 μM PAR-APs (GYPGQV: PAR-4AP, SFLLRN: PAR-1AP). Immunodetection of phosphorylated ERKs (ERK-P) and total ERKs (ERK-tot) after stripping and reprobing the same membrane.

induced calcium mobilization contrasts with the delayed effect on apoptosis, consistent with the fact that PAR-1-associated signaling is not required for thrombin-induced apoptosis in our model. The kinetics of PAR-1/4-coupled intracellular signaling were also evaluated at the level of ERK-1/2 phosphorylation (Fig. 2C). Thrombin (100 nM) and both PAR-APs (250 μM) were able to trigger ERK phosphorylation in human VSMCs. In contrast to what has been observed on microglial cells [26], PAR-4 AP did not prolong ERK activation beyond 2 h even in the presence of concentrations of GYPGQV higher than 250 μM (not shown). These results indicate that thrombin induces apoptosis of VSMCs independently of the activation of PARs. This was further supported by the fact that thrombin-induced DNA fragmentation was not inhibited by PD98059, an inhibitor of ERK activation. Finally, the addition of hirudin 2 h after thrombin, that is, after the PARs-coupled pathways have been activated, thwarted the pro-apoptotic effect of thrombin. Altogether, these results exclude the involvement of PARs in our model and suggest that the mechanism leading to apoptosis relies on a sustained proteolytic effect of thrombin.

Thrombin-induced degradation of the extracellular matrix

VSMC retraction (Fig. 3A) paralleled the pro-apoptotic effect of thrombin, suggesting an extracellular proteolytic effect. In contrast, these thrombin-induced effects were not mimicked by the PAR-1 agonist, SFLLRN, up to 1000 μM (not shown). Since intact fibronectin is considered as a survival factor for adherent cells [27] and particularly SMCs [28], and thrombin is capable of cleaving fibronectin [9,29], we hypothesized that fibronectin could be proteolyzed in our experimental conditions. We observed a cleavage of cellular fibronectin that paralleled the pro-apoptotic effect of thrombin, and was inhibited in the presence of hirudin or PN-1 (Fig. 3B). The inhibition by PN-1 of thrombin-induced SMC apoptosis and of fibronectin degradation is consistent with the observation that PN-1 is expressed in the arterial wall [22] and is able to prevent pericellular proteolysis and subsequent apoptosis of adherent cells. The degradation of the extracellular matrix was further illustrated by the solubilization of TGF-β1, which was detected in the conditioned medium of thrombin-treated VSMCs [30] (Fig. 3C).

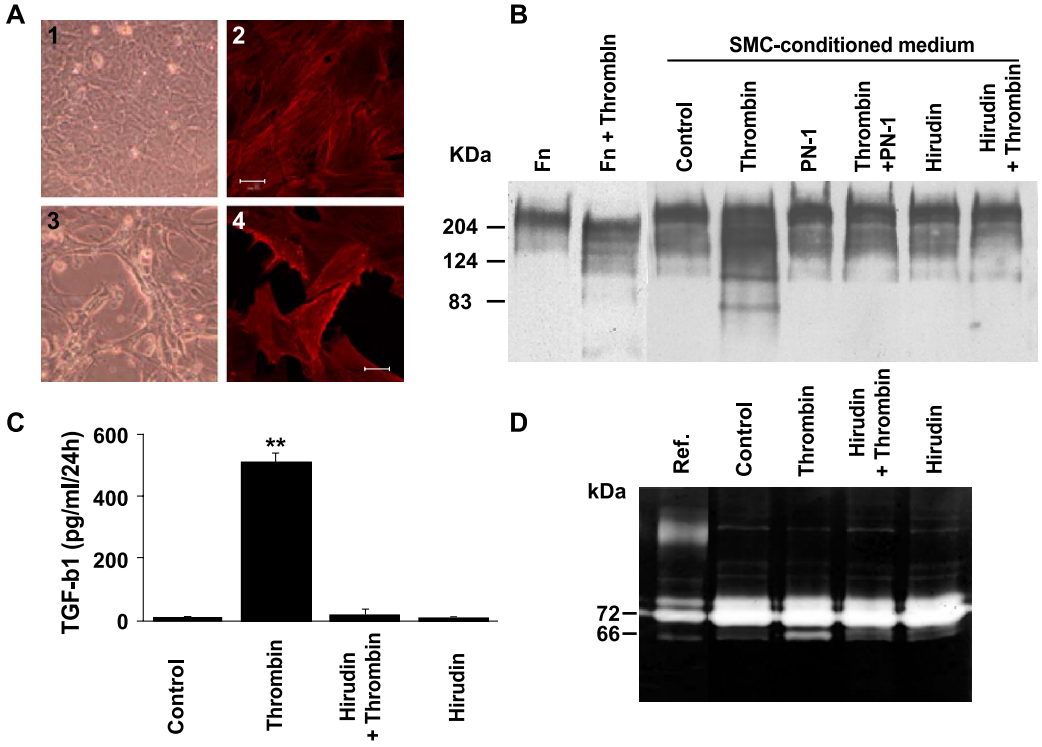

Fig. 3. Thrombin-induced cell retraction and pericellular proteolysis parallel its pro-apoptotic effect. (A) Rat VSMC retraction: inverse phase (A1, 3) and α-actin immunofluorescence detection by confocal microscopy (A2, 4) of rat VSMCs incubated for 48 h without (A1–2), or with 100 nM thrombin (A3, 4). (B) Fibronectin proteolysis by thrombin. Western blot probed with an anti-fibronectin antibody detected an in vitro degradation pattern of purified human fibronectin (Fn) when incubated with 100 nM thrombin for 72 h at 37 °C (Fn + thrombin). Similar degradation patterns were observed in the conditioned media of VSMCs incubated with 100 nM thrombin for 72 h. PN-1 (200 nM) and hirudin (1 μM) inhibited thrombin-mediated degradation of fibronectin. (C) ELISA quantification of solubilized TGF-β1 in rat aortic SMC conditioned media. Results are expressed in pg of TGF-β1/ml of medium conditioned during 24 h of incubation by about 50,000 cells (mean of four separate experiments ± SD, $^{**}P < 0.01$ thrombin vs. all other conditions). (D) Gelatin zymography showing the presence of active MMP-2 in conditioned media of VSMCs treated by thrombin (100 nM) for 72 h. Hirudin (1 μM) prevented activation of pro-MMP-2 by thrombin.

At low concentrations, thrombin has been shown to induce endothelial cell retraction and increase the monolayer permeability (for review, see Ref. [31]) with a major reorganization of the actin cytoskeleton, the formation of blebs and endothelial cell rounding without evidence of apoptosis [32]. Cytochalasin D (40 μM for 72 h), used as an actin filament-disrupting agent [33], induced marked cell rounding accompanied by apoptosis. Combined effects of cytochalasin D (intracellular actin network disorganization) and thrombin (proteolysis of extracellular matrix cell anchorage) potentiated apoptosis (DNA fragmentation: cytochalasin D + 100 nM thrombin: 53.5 ± 18.6 vs. 24.5 ± 0.8 A_{405nm} 10^{-3} min^{-1} for cytochalasin alone). These results suggest that loss of tensional integrity [34] due to cytoskeletal disorganization may be involved in the pro-apoptotic effects of thrombin on VSMCs. Because VSMCs secrete promatrix metalloproteinase-2 (MMP-2) in large amount, we investigated its role in our model, since thrombin is able to convert pro- into active MMP-2 [13,35]. We show that medium from thrombin-treated cells contains the active form of MMP-2 (Fig. 3D). However, GM6001 (a broad range MMP inhibitor used at 10 μM) failed to prevent apoptosis, arguing for a minor role of the MMPs in our apoptosis model. Moreover, conditioned media of apoptotic thrombin-treated VSMCs (containing active MMP-2), in which thrombin was neutralized by hirudin, did not mimic the pro-apoptotic effects of thrombin, suggesting that thrombin exerted its pericellular proteolytic pro-apoptotic effects directly (not shown). In this proteolytic context, we examined whether degradation of thrombin occurs during incubation. This could expose its RGD-containing motif [36], which would then compete with integrin–matrix interactions. Immunodetection by Western blot analysis showed that α-thrombin integrity was preserved for over 72 h of incubation, excluding the possibility that competition between thrombin and integrins could account for cell apoptosis in this model (not shown).

Our data, in agreement with previous results obtained in neurons or tumor cell lines, demonstrate a pro-apoptotic effect of thrombin on confluent VSMCs, requiring both higher concentrations of thrombin than those required for mitogenesis and a more prolonged exposure (at least 24 h). However, in contrast to observations obtained with most other cells, the pro-apoptotic effect of thrombin on VSMCs is PAR (1 and 4)-independent. Interestingly, apoptosis induced by thrombin was observed later in human VSMCs than in rat aortic VSMCs (96 versus 24–72 h). This could be explained by the more important synthesis of extracellular matrix by human VSMCs, which take more time to reach confluence than rat VSMCs. Our results suggest that an extracellular pathway may be involved in the pro-apoptotic effect of thrombin on VSMCs, with parallel proteolytic cleavage of fibronectin, cytoskeletal disorganization, and cell retraction, causing disruption of anchorage-dependent survival signaling. Such a phenomenon has already been described by our group [37] using VSMCs incubated with other proteases able to cleave adhesive glycoproteins, such as elastase [21] and plasmin [38] and by others using cathepsin G on cardiomyocytes [39].

In conclusion, our results demonstrate that beyond its mitogenic effect, thrombin at high concentrations exhibits a pro-apoptotic effect on VSMCs that involves its proteolytic activity. This effect is not receptor-mediated. The parallel cleavage of fibronectin suggests that thrombin-induced apoptosis is consecutive to pericellular proteolysis. The in vivo relevance of this phenomenon in vascular pathologies involving thrombin formation and SMC disappearance remains to be elucidated.

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