Role of Leukocyte Elastase in Preventing Cellular Re-Colonization of the Mural Thrombus

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To explore possible mechanisms responsible for the absence of cell re-colonization of mural thrombi in aneurysms, we analyzed the release and storage of leukocyte proteases in the most luminal layer *versus* intermediate and abluminal layers of 10 mural thrombi of human abdominal aortic aneurysms. The luminal layer contained many polymorphonuclear leukocytes (PMNs), which released pro-matrix metalloproteinase (MMP)-9 and MMP-8. Leukocyte elastase was also stored and released by the luminal layer (immunohistochemistry, activity on synthetic substrates, and casein zymography). Acid buffer allowed extraction of leukocyte elastase from the luminal layer, which was inhibited by elastase inhibitors. Casein zymography of luminal extracts and conditioned medium from formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated PMNs exhibited a similar lysis pattern, corresponding to elastase activity. Smooth muscle cell (SMC) seeding resulted in colonization of the intermediate thrombus layer *ex vivo* but not of the luminal layer. Extracts of the luminal layer induced loss of anchorage of both cultured human smooth muscle cells and stromal cells of bone marrow origin (anoikis). This anoikis was prevented by preincubation of the extracts with serine protease inhibitors. Moreover, adhesion of human SMCs and stromal bone marrow cells on fibrin gels was strongly inhibited when the gel was preincubated with pure elastase, medium of fMLP-stimulated PMNs, or extracts of luminal layers of mural thrombi. This loss of cell anchorage was prevented by the preincubation of the medium or extracts with α_1 -antitrypsin, but not when α_1 -antitrypsin was added after binding of elastase to the fibrin gel. In conclusion, elastase released by PMNs trapped within the mural thrombus impairs

the spontaneous anchorage of mesenchymal cells to a fibrin matrix. This phenomenon could be one mechanism by which cellular healing of the mural thrombus in aneurysms is prevented. (Am J Pathol 2004, 164:2077–2087)

In response to tissue injury, a provisional matrix, largely composed of cross-linked fibrin and fibronectin, is deposited at the affected site.¹ This fibrin-fibronectin scaffolding functions initially as a structural support for infiltrating cells, leading to cell re-colonization and subsequent tissue healing.^{2–4} Fibrin polymerization favors the adhesion of many cell types, including inflammatory cells,^{5,6} epithelial cells,⁷ endothelial cells,^{8,9} and fibroblasts.¹⁰ Cell adhesion to fibrin matrices is mediated by several molecules including cell adhesion molecules,¹¹ cadherins,^{8,9} and arginine glycine aspartic acid (RGD)-binding integrins.¹² In particular, polymorphonuclear leukocytes (PMNs) adhere to fibrin via the β_2 -integrins and L-selectin,⁵ whereas smooth muscle cells (SMCs) colonize fibrin gels via $\alpha_v\beta_3$ -integrins and intercellular adhesion molecule (ICAM)-1.¹³ Fibronectin is the main adhesive protein¹⁴ involved in the survival of adherent cells.^{15,16} Cellular fibronectin is synthesized and secreted by SMCs, which adhere to it¹⁷ and by adherent stromal cells of bone marrow origin.¹⁸ Plasma fibronectin, synthesized and secreted by the liver, binds to fibrin during clotting and to collagen and heparan sulfates in tissues.

Contrasting with the healing properties of the fibrin-fibronectin matrix in other tissues, the fibrin matrix of aneurysmal mural thrombi, which maintains a continuous interface with circulating blood, is not colonized by adherent cells, allowing the proteolytic injury of the arterial wall to continue unimpeded, leading to dilatation and rupture.¹⁹ Whereas the common response of the arterial wall to injury is intimal colonization,²⁰ aneurysms are characterized by extracellular matrix degradation, disappearance of SMCs, presence of a mural thrombus, and absence of colonization by mesenchymal cells.¹⁹ In stenosing atherothrombosis, SMC proliferation, matrix production, and re-endothelialization can lead to incorpora-

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Figure 1. Release of MMP-9 and MMP-8 by different layers of the mural thrombus. **A:** A macroscopic example of an aneurysmal thrombus showing the luminal, intermediate, and abluminal layers. **B:** MMP-9 and MMP-2 activities released by the layers of the aneurysmal thrombus ($n = 10$). **C:** Neutrophil collagenase (ELISA anti-MMP-8) released by the three layers ($n = 10$). ** $P < 0.01$, luminal versus intermediate and abluminal.

tion of a mural thrombus into the arterial wall, a phenomenon which appears not to occur in abdominal aortic aneurysms. Mesenchymal cell colonization of the intima may result predominantly from migration of SMCs from the media, but also from adhesion of circulating progenitors of bone marrow origin at the site of injury.²¹ We have recently shown that, compared to the deeper layers, the luminal layer of the aneurysmal thrombus is enriched with PMNs and releases gelatinase B (MMP-9).²² In parallel, it has been reported that elastase released by PMN degranulation is able to induce loss of cell anchorage and apoptosis (anoikis) of cultured adherent cells.^{19,23} It thus appears probable that elastase released by PMNs trapped within the fibrin matrix may play a role in preventing the colonization of the mural thrombus by mesenchymal cells. To test this hypothesis, we analyzed first the release and storage of neutrophil proteases by human aneurysmal thrombi *ex vivo*. Since cultured adherent bone marrow stromal cells (BMSCs) spontaneously differentiate into SMC-like mesenchymal cells,^{18,24} we also investigated the effect of elastase released by PMNs and bound to fibrin on the ability of both somatic SMCs and BMSCs to colonize a fibrin matrix.

Materials and Methods

Ex Vivo Studies of Aneurysmal Thrombus

Ten mural thrombi from human aneurysms were collected during surgical repair and rapidly dissected into three parts: the luminal part, the intermediate part, and the abluminal fibrinolytic part (Figure 1A). The samples were obtained as surgical residues in accordance with the French ethical laws (L.1211-3-9).

Using a method we have already proposed for the discovery of new secreted biological markers of atherosclerosis,²⁵ each layer was cut into small pieces (1 mm³) and separately incubated in RPMI 1640 medium (Gibco, Cergy-Pontoise, France) for 24 hours at 37°C (2 ml/g of wet tissue). The conditioned media were then used for determination of secreted protease activities and for demonstrating the presence of solubilized fibronectin fragments.

As previously demonstrated,²⁶ elastase was extracted from each layer by agitation with 1 mol/L acetate buffer, pH 4.5 (2 ml/g of wet tissue), for 2 hours at room temperature. Extracts were then dialyzed against 50 mmol/L Tris-HCl pH 7.5, 0.2% Triton X-100, or phosphate-buffered saline (PBS) for activity and culture assays, respectively. Protein concentrations were determined by the Bradford assay (Bio-Rad, Marne la Coquette, France).

For examination by light microscopy, small samples of the three layers of mural thrombi were fixed in 3.7% paraformaldehyde and embedded in paraffin. Serial 5- μ m sections were used for nuclear staining and immunohistochemistry. Hematoxylin/eosin was used to show cell nuclei within the thrombus. Neutrophil elastase was immunolocalized by a specific anti-human elastase antibody (Calbiochem, Nottingham, England) used at 1:200 dilution, smooth muscle cell by α -actin antibody (DAKO, Trappes, France) used at 1:50 dilution and revealed by the peroxidase-DAB method (Vectastain ABC kit, Vector, Paris, France). Human PMNs were used as positive controls. A negative control was obtained by the preincubation of the antibodies with pure leukocyte elastase.

To test the ability of the different parts of the thrombus to be colonized by SMCs, thin slices (100 μ m) of luminal and intermediate layers of fresh thrombi (~ 2 cm²) were sampled and placed flat on the well bottom in 6-well plates. These slices covered approximately half of the surface of the well bottom. Smooth muscle cells were then seeded at a density of 5×10^5 cells per well and colonization of both thrombus slices and of the surrounding plastic well surface were analyzed 1 week later. The thrombus slice was removed, fixed, and embedded for light microscopy as described above. Five- μ m sections were stained with hematoxylin/eosin and SMC α -actin antibody. SMCs remaining in the well were also stained with hematoxylin/eosin and examined *in situ* by phase contrast microscopy.

In Vitro Studies of SMC-PMN-Fibrin Interactions

Human SMCs were obtained from small pieces of human radial and mammary arteries by explants of the medial layer.^{27,28} SMCs were cultured in SMC basal medium 2 (Promocell, Heidelberg, Germany) containing 10% fetal calf serum (FCS), gentamicin (25 μ g/ml), amphotericin (25 ng/ml), insulin (2.5 μ g/ml), human fibroblast growth factor (hFGF) (1 ng/ml), and human epithelial growth factor (hEGF) (0.25 ng/ml). Cultures were performed at 37°C, 5% CO₂. Cells were used at passage 4.

Stromal cells of bone marrow origin were isolated and cultured as previously described^{18,24} with slight modifications. Briefly, bone marrow was extracted from femoral heads obtained during surgical repair. Bone marrow cells were eluted by Hanks' balanced salt solution (HBSS) flush and residual erythrocytes were eliminated by hypo-osmotic shock. Cells eluted from marrow were then seeded in tissue culture flasks coated with fibronectin (50 $\mu\text{g/ml}$) and cultured in SMC basal medium 2 (Promocell) containing 10% FCS, gentamicin (25 $\mu\text{g/ml}$), amphotericin (25 ng/ml), insulin (2.5 $\mu\text{g/ml}$), hbFGF (1 ng/ml), and hEGF (0.25 ng/ml). The medium was completely replaced every 2 days and non-adherent cells were discarded. Confluency was obtained after 1 month of culture. Cultures were performed at 37°C, 5% CO₂. Cells were used at passage 2.

PMNs were obtained from heparinized venous blood of volunteers by 2% dextran sedimentation followed by Ficoll-Paque (Amersham, Piscataway, NJ, USA) centrifugation and hypo-osmotic lysis of residual erythrocytes.²³ Cells were maintained in HBSS. The viability, measured by the release of lactate dehydrogenase (LDH)²⁹ and purity of the final preparation were greater than 99 and 95%, respectively. PMN degranulation was obtained by addition of formyl-methionyl-leucyl-phenylalanine (fMLP) at 10⁻⁶ M for 10 minutes, at 37°C (500 μl of conditioned medium corresponding to the release of 5 $\times$ 10⁵ activated PMN were used throughout the experimental procedure).

Twenty-four- and 96-well plates were coated overnight with human fibrinogen (LFB) at 5 mg/ml at room temperature. Wells were washed with PBS and 2 U/ml thrombin in PBS (Stago, Parnippany, NJ, USA) were added and incubated for 2 hours at 37°C. Wells were rinsed and residual thrombin activity was inhibited by addition of 1 $\mu\text{mol/L}$ PPACK (Bachem, Western Rhein, Germany). Wells were rinsed again with PBS and stored in the same buffer at 4°C until use.

Elastase, activated-PMN supernatants, and mural thrombus extracts, alone or treated with different inhibitors: 2 mmol/L PMSF (serine protease inhibitor), 10⁻⁵ M PD0166973 (matrix metalloprotease inhibitor) (Parke Davis), 10⁻⁶ M SLPI (secretory leukocyte protease inhibitor) (Generous gift of Michel Chignard and Dominique Pidard, Pasteur Institute, Paris)^{30,31} or 10⁻⁷ M α_1 -antitrypsin (serine protease inhibitor) (Calbiochem) were incubated on 24-well plates coated with fibrin overnight. Wells were rinsed with PBS buffer and human SMCs or BMSCs were loaded (2.5 $\times$ 10⁵/wells) in basal medium with gentamicin (25 $\mu\text{g/ml}$), amphotericin (25 ng/ml), hbFGF (1 ng/ml), and hEGF (0.25 ng/ml). After 24 hours of incubation, cells were photographed, supernatants were collected and frozen until use and cell viability was determined.

Cells were washed with PBS. Remaining viable adherent cells were quantified using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) test (Sigma, Lyon, France).³² Cells were incubated for 1 hour with PBS containing 0.5 mg/ml of MTT at 37°C. After aspiration, remaining formazan crystals were dissolved in dimethyl sulfoxide and homogenized. Absorbance was

read at 550 nm with a microtitration plate reader (Titertek). All of these tests were performed in 24-well plates.

The method of terminal transferase-mediated dUTP nick end-labeling (TUNEL) was used to visualize DNA fragmentation.³³ A cell death detection kit (Roche, Basel, Switzerland) was used according to the manufacturer's instructions. A positive control (1 $\mu\text{g/ml}$ DNase I treatment for 10 minutes after permeabilization) and a negative control (without terminal transferase) were included in each set of experiments. Total cell nuclei were counterstained with 100 ng/ml DAPI (4',6'-diamidino-2-phenylindole hydrochloride).

Genomic DNA was isolated from SMCs and BMSCs using standard DNA extraction methods (G-NOME kit, BI0101). DNA (10 μg) was loaded on a 1.8% agarose gel containing 0.5 mg/ml ethidium bromide and separated by electrophoresis allowing detection of DNA fragmentation (DNA ladder).³⁴

Cell death detection was performed by photometric enzyme immunoassay for *in vitro* determination of cytoplasmic histone-associated DNA fragments according to the manufacturer's instructions (Roche).

Non-adherent cells were centrifuged at 150 $\times g$ for 6 minutes onto slides, fixed with cold methanol for 5 minutes or paraformaldehyde for 1 hour, then stained with hematoxylin and eosin before microscopic observation or used for TUNEL assay or DNA ladder electrophoresis.

Proteolytic Activities

PMN media alone or treated with different inhibitors (2 mmol/L PMSF, 10⁻⁵ M PD0166973, 10⁻⁶ M SLPI, 10⁻⁷ M α_1 -antitrypsin) were incubated overnight in 96-well plates coated with fibrin. Wells were rinsed with PBS before determination of elastase activity with a specific chromogenic substrate, 1.5 mmol/L N-Met-Suc-Ala-Ala-Pro-Val-pNA (Sigma).³⁵ The release of human MMP-8 antigen was measured in the conditioned media using a commercially available Biotrak ELISA system (Amersham).

Gelatinolytic and caseinolytic activities in tissue extracts and conditioned media were measured as previously described.²² Samples containing 5 μg and 15 μg of proteins were loaded on gelatin and casein gels, respectively. Gelatin and casein gels were incubated at 37°C for 19 hours and 72 hours, respectively. Lysis areas were quantified by densitometric scanning using NIH Image 1.61 software. To discriminate between MMP and serine protease activity, additional gels were incubated in presence of inhibitors of MMPs (30 mmol/L EDTA) or serine proteases (2 mmol/L Pefabloc).

Standard procedures were used for Western blot analysis.³⁶ Fibronectin was detected in conditioned media using SDS-PAGE, and proteins were transferred to PVDF membranes (NEN, Monza, Italy). The membranes were blotted with anti-fibronectin antibody (Ab-1, 1:2000 dilution; Calbiochem) and a peroxidase-conjugated secondary antibody. Membranes were then exposed to X-ray films for chemiluminescence detection. Purified human fibronectin was purchased from Sigma and used as control. Fibronectin degradation was obtained by incubation

of fibronectin with activated PMN-conditioned media (1 μ g of fibronectin with 500 μ l of activated PMN-conditioned media (10^6 PMN/ml)) for 1 hour at 37°C.

Statistical Analysis

Each experiment was performed on at least three samples, in triplicate. Data are presented as means $\pm$ SD. Analysis was performed by paired tests for comparison of luminal versus intermediate and abluminal parameters, and by a one-way analysis of variance followed by the Fisher test, when appropriate. A value of $P < 0.05$ was considered statistically significant.

Results

Storage and Release of Elastase by the Luminal Layer of the Mural Thrombus

All aneurysmal mural thrombi were composed of three layers with a fresh clot at the luminal surface (Figure 1A). Microscopic observation after hematoxylin/ eosin staining demonstrated the presence of numerous polymorphonuclear leukocytes in this layer, as shown by the polylobed aspect of the cell nuclei (Figure 2A). Gelatin zymography, performed on the conditioned media after 24 hours incubation of the different layers of the thrombus, provided evidence of the release of MMP-9 and MMP-2 by the thrombus. The MMP-9 is probably, at least in part, of PMN origin as demonstrated by the presence of a 125-kd band of lysis in the luminal layer corresponding to the MMP-9-lipocalin heterodimer complex, secreted by PMNs (Figure 1B).³⁷ Release of MMP-8 antigen (neutrophil collagenase)³⁸ also predominated in the luminal layer (Figure 1C).

We then investigated whether elastase was released by the thrombus into the tissue culture-conditioned medium or whether it was stored within the fibrin polymer. The different layers of the thrombus were incubated with various buffers of different ranges of molarity and pH. Incubation with 1mol/L acetate buffer at pH 4.8 for 2 hours allowed us to recover elastase activity trapped in the fibrin clot, as demonstrated by the use of a specific chromogenic substrate of leukocyte elastase. This activity was detected mainly in the extract of the luminal layer of the thrombus, confirming the presence of leukocyte elastase in this layer (Figure 2B). It was inhibited by PMSF and α_1 -antitrypsin but not by PD0166973, an MMP inhibitor (Figure 2C). These data were confirmed by detection of a caseinolytic activity at 28 kd mainly in extracts of the luminal layer of the thrombus (15 μ g of total proteins loaded on gel), corresponding to the elastase activity released by f-MLP-activated PMNs (Figure 2D). Only low levels of elastase activity were detected in neutral-conditioned media of the luminal layer (100 μ g of total proteins loaded on gel), an activity which was 10-fold lower than that in acidic luminal extracts (Figure 2D), indicating that most elastase activity in the luminal thrombus remains bound to the fibrin matrix.

Figure 2. Neutrophil elastase in the luminal layer of the mural thrombus. **A:** Co-localization of PMNs and elastase: **Top**, Hematoxylin/eosin/safran staining showing the presence of PMNs in the luminal pole of the thrombus ($\times 10$) and **inset** ($\times 100$). **Bottom**, Neutrophil elastase staining in the luminal pole of the thrombus ($\times 10$), and **inset** ($\times 100$) confirming the co-localization of neutrophil elastase and PMNs. **B–D:** Biochemical evidence of elastase within the luminal part of 10 thrombi. **B:** Elastase activity was detected using a specific chromogenic substrate (N-Met-Suc-Ala-Ala-Pro-Val-pNA). Elastase activity largely predominated in the luminal extracts of aneurysmal thrombus. *** $P < 0.001$, luminal versus intermediate and abluminal. **C:** Inhibitory profile of the elastase activity detected in luminal extracts. *** $P < 0.001$, control and MMP inhibitor (MMPI) versus PMSF and α_1 -antitrypsin. **D:** Caseinolytic activity at 28 kd detected in three acidic extracts (15 μ g of proteins) and neutral-conditioned media (100 μ g of proteins) of aneurysmal thrombus corresponding to leukocyte elastase. This lytic band was inhibited by Pefabloc 2 mmol/L (serine protease inhibitor) but not by EDTA 30 mmol/L (MMP inhibitor) (data not shown). **E:** Western blot of fibronectin in conditioned media of mural thrombi (columns 1, 2, and 3) showed the presence of proteolytic degradation products of fibronectin: column 4, purified fibronectin; column 5, purified fibronectin preincubated with activated PMN-conditioned medium (500 μ l of PMN-conditioned medium (10^6 PMN/ml for 1 μ g of fibronectin, 1 hour of incubation at 37°C)) serve as non-degraded and degraded fibronectin controls, respectively.

Neutrophil Elastase Localization

Immunohistochemistry revealed the presence of elastase in polymorphonuclear leukocytes. Moreover positive staining was not restricted to the cells but was present also in the fibrin matrix surrounding dying cells (Figure 2A).

Fibronectin Fragmentation Was Present in Thrombus Extracts

Because leukocyte elastase released from activated PMNs is able to degrade fibronectin,^{23,39} Figure 2E

Figure 3. Extracts of luminal thrombi induced anoikis of cultured SMCs. **A:** Incubation of cultured SMCs with extracts of the luminal layer of the thrombus induced SMC loss of anchorage and apoptosis. This phenomenon was prevented by preincubation of the conditioned medium with α_1 -antitrypsin (α_1 -AT). In contrast, extracts from the abluminal layer did not induce apoptosis. **B:** These morphological data are confirmed by quantification of cell viability using the MTT test ($n = 15$). *** $P < 0.001$, luminal *versus* luminal + α_1 -antitrypsin (α_1 -AT) and abluminal. **C:** These morphological data are also confirmed by demonstration of DNA laddering.

shows the presence of fibronectin fragments, spontaneously released by the thrombus in the conditioned medium.

Extracts of Luminal Thrombus Induce Anoikis of SMCs and BMSCs in Culture

As we have previously demonstrated that leukocyte elastase released by activated PMNs induced anoikis of SMCs,²³ the hypothesis that extracts from luminal thrombus containing leukocyte elastase can induce a similar phenomenon was tested. Incubation of cultured SMCs with extracts of luminal thrombus induced cell retraction and detachment (Figure 3B) which did not occur with extracts of the abluminal part of the thrombus or with control buffer. This cell retraction was associated with TUNEL positivity (Figure 3A) and DNA laddering (Figure 3C). SMC anoikis was prevented by preincubation of luminal extracts with α_1 -antitrypsin (Figure 3, A to C). Similar results were obtained with human BMSCs (Figure 4, A and B). Incubation of stromal cells with PMN medium, luminal extract, and purified elastase induced cell retraction and detachment. This phenomenon was prevented by the pretreatment of PMN medium with α_1 -

antitrypsin and SLPI inhibitors. Western blot analysis showed the presence of fibronectin degradation fragments in conditioned media of cultured stromal cells treated with PMN medium, luminal extract, or purified elastase. This degradation was partially prevented with addition of α_1 -antitrypsin and SLPI before incubation (Figure 4C). Stromal cells treated with PMN medium, elastase, or luminal extract presented marked DNA fragmentation that was prevented by preincubation of PMN media with α_1 -antitrypsin and SLPI (Figure 4D).

These results were confirmed *ex vivo* by seeding thin slices of the luminal and intermediate layers of the thrombus with SMCs ($n = 5$) (Figure 5). Seven days later, SMCs had grown to confluency on the plastic surface surrounding the thrombus slices in wells containing the intermediate layer whereas in the wells containing the luminal layers, the SMCs were less confluent (Figure 5A). Similarly SMCs had grown and colonized the surface of the slices of the thrombus intermediate layer, whereas no SMCs were detectable on the surface of the luminal slices (Figure 5B).

Storage of Leukocyte Elastase within a Fibrin Matrix Prevented Its Colonization by SMCs and BMSCs in Vitro

Neutrophil Elastase Activity Is Detected on the Fibrin Matrix after Exposure to PMN Medium

A chromogenic substrate and specific inhibitors were used to measure and characterize the binding of elastase activity to a fibrin matrix. Neutrophil elastase activity was detectable on fibrin matrices preincubated for 12 hours with PMN-conditioned medium and washed three times with PBS buffer (Figure 6A). This activity was inhibited by 2 mmol/L PMSF, a serine protease inhibitor, and by SLPI and α_1 -antitrypsin which are specific inhibitors of neutrophil elastase (Figure 6A). Similar results were obtained when preincubation was performed with luminal extracts of mural thrombus.

SMCs and BMSCs Can Colonize a Fibrin Matrix

The ability of SMCs to adhere to a fibrin matrix was checked first. For this purpose, different numbers of smooth muscle cells, varying from 25,000 to 250,000 cells per well, were seeded in 24-well plates to quantify the percentage of cell adhesion. Smooth muscle cells adhered to the fibrin matrix at $91 \pm 2\%$. After seeding of 2.5×10^5 SMCs per well, confluency was reached after 24 hours. Similar results were obtained with BMSCs, which also reached confluency after 24 hours of culture.

Preincubation of a Fibrin Matrix with PMN-Conditioned Media Prevents SMC and Mesenchymal Stem Cell Adhesion and Survival

When fibrin matrices were preincubated with PMN supernatant, SMC adhesion was significantly decreased compared to non-preincubated fibrin matrices (Figure

Figure 4. Extracts of PMN medium and luminal thrombi induced anoikis of stromal cells of bone marrow origin. **A:** Phase contrast photomicrographs of BMSCs in culture. **A and B:** Incubation of stromal cells with PMN medium (PMN) and luminal extract (luminal) ($n = 5$) induced stromal cell loss of anchorage. This phenomenon was prevented by preincubation with α_1 -antitrypsin (α_1 -AT) and SLPI of both PMN releasates and luminal extracts of mural thrombus. *** $P < 0.001$, α_1 -AT and SLPI *versus* PMN medium, purified leukocyte elastase, and luminal thrombus extract. **C:** Western blot of conditioned media of cultured stromal cells treated with PMN medium, luminal thrombus extract, and purified leukocyte elastase showing first the ability of adherent BMSCs to synthesize their own fibronectin¹⁸ and secondly the degradation of fibronectin by PMN secretion, luminal thrombus extract, and elastase. Preincubation of luminal thrombus extracts with α_1 -AT and SLPI prevent fibronectin degradation. **D:** Quantification of DNA fragmentation in cultured BMSCs treated with PMN medium, luminal thrombus extract, and purified leukocyte elastase. Preincubation of PMN medium and luminal thrombus extracts with α_1 -AT and SLPI prevent DNA fragmentation. *** $P < 0.001$, α_1 -antitrypsin and SLPI *versus* PMN medium, purified leukocyte elastase, and luminal thrombus extract.

6B). Cell adhesion and viability remained at control levels when PMN secretion products were preincubated with specific inhibitors of serine proteases and neutrophil elastase ($P < 0.001$) whereas an MMP inhibitor had no effect. The elastase activity associated with these fibrin gels was inversely proportional to the cell viability ($P < 0.001$, Figure 6B). In contrast, when α_1 -antitrypsin was added after elastase had bound to the fibrin gel, this natural inhibitor did not prevent smooth muscle cell loss of anchorage to fibrin (percentage of adherent cells: α_1 -antitrypsin preincubation: $90 \pm 4\%$ *versus* α_1 -antitrypsin post-incubation: $25 \pm 5\%$ ($P < 0.01$)). Similarly, preincubation of the fibrin gel with PMN medium or extracts of the luminal layer of aneurysmal thrombus, prevented BMSC anchorage to fibrin (Figure 7, A and B). This effect was reversed by preincubation with elastase inhibitors.

Cell Morphology and Apoptosis of Adherent and Detached SMCs and BMSCs

Hematoxylin/eosin staining, DAPI, and TUNEL reaction for detection of apoptosis were applied to adherent and detached SMCs (Figure 6C). Both hematoxylin/eosin

stain and DAPI showed a uniform staining of SMCs with no nuclear condensation when SMCs were directly seeded on fibrin (data not shown). Fibrin preincubated with elastase, PMN-conditioned media, or with extracts of the luminal layer of the thrombus, did not allow SMC adhesion. Detached cells remaining in the supernatant and residual cells on fibrin exhibited nuclear condensation and were positive for the TUNEL assay. The use of α_1 -antitrypsin prevented SMC detachment and nuclear condensation and cells remained negative for the TUNEL assay. Detached SMCs obtained after preincubation of the fibrin matrix with PMN secretion products or luminal layer extracts of thrombus presented nuclear condensation and were TUNEL positive. Similar results were obtained with BMSCs. Treatment of the fibrin matrix with PMN medium, luminal extract, and elastase also impaired BMSC adhesion (Figure 7, A to C). This phenomenon was prevented by incubation of the media with α_1 -antitrypsin and SLPI before adsorption to fibrin (Figure 7, B and C). BMSCs adhering to fibrin pretreated with PMN medium or luminal extract showed marked DNA fragmentation. The use of α_1 -antitrypsin and SPLI inhibitors prevented this fragmentation (Figure 7, D and E).

A

Colonization of the well

B

Colonization of the thrombus slices

Figure 5. SMCs did not re-colonize the luminal thrombus surface *ex vivo*. **A:** Phase contrast micrographs of SMC colonization of the part of the well surrounding a slice of the luminal or intermediate part of the thrombus 7 days after seeding, showing total colonization in wells containing intermediate slices and an incomplete colonization in wells containing luminal slices. **B:** Staining of SMCs (hematoxylin/eosin and α -actin) on the surface of the thrombus slices: SMCs were undetectable on the luminal slices whereas they colonize the intermediate slices (negative controls not shown).

Discussion

Contrasting with other pathological situations involving fibrin formation in the healing process, mural thrombi in aneurysms are characterized by an absence of healing and cell colonization. This absence of cellular healing is one of the main features differentiating aneurysmal disease from other forms of arterial wall remodeling in response to atheromatous injury. One of the processes of atherosclerotic plaque evolution toward stenosis involves plaque rupture, formation of a thrombus and its incorporation within the arterial wall in the course of cellular healing.⁴⁰ In contrast, aneurysmal evolution toward increased dilatation leading to rupture is not associated

with cellular healing and, moreover, the seeding of experimental aneurysms with SMCs has been shown to prevent arterial dilatation and rupture.^{41,42}

In a recent study, we have shown that the mural thrombus in human atherosclerotic aneurysms is a site of storage and release of MMP-9 and that polymorphonuclear leukocytes trapped within the thrombus are the main cellular source of this MMP-9.²² Recent luminal clots are sites of platelet deposition⁴³ and fibrin-fibronectin copolymerization,⁴⁴ and represent an optimal substrate for PMN adhesion. In the present work, we have extended these data using an *ex vivo* approach to study proteases bound to and released by the mural thrombus in human aneurysms. Moreover, to explore in more detail the topol-

Figure 6. Viability of adherent smooth muscle cells and elastase activity on fibrin matrices. **A:** Elastase activity bound to fibrin matrix, preincubated with PMN media (500 μ l of conditioned medium corresponding to 5.10^5 activated PMN) and luminal extracts ($n = 5$) alone or with different elastase inhibitors, was determined using a specific chromogenic substrate (N-Met-Suc-Ala-Ala-Pro-Val-pNA). Preincubation with elastase inhibitors effectively reduced this activity and restored SMC colonization. Incubation of the fibrin matrix with luminal extracts of mural thrombus gave similar results. *** $P < 0.001$. **B:** Percentage of viable adherent SMCs was determined using the MTT test after incubation of $2.5.10^5$ SMCs on a fibrin matrix preincubated or not with PMN-conditioned media (500 μ l of fMLP 10^{-6} M-stimulated PMN secretion corresponding to 5.10^5 PMN) and luminal extracts associated or not with inhibitors: PMSF, MMP inhibitor, SLPI, and α_1 -antitrypsin (α_1 -AT). *** $P < 0.001$. **C:** Characterization of cells adhering to or detached from fibrin matrices preincubated with activated PMN secretion products: adhesion was poor and cells were retracted and apoptotic (TUNEL+) indicating that anoikis had occurred. The majority of cells did not adhere. Addition of α_1 -antitrypsin to PMN secretion products prevented anoikis of SMCs.

ogy of mural thrombus homeostasis, involving both the luminal interface with the circulating blood⁴⁵ and the abluminal interface with the aneurysmal wall,²² fresh mural thrombi were dissected into three layers: luminal, corresponding to the most recent thrombus layer, intermediate, and abluminal, corresponding to the fibrinolytic interface with the aneurysmal wall. Our data confirm that the thrombus can release pro-MMP-9 and provide new evidence that MMP-9 comes, at least in part, from fibrin-trapped PMNs within the luminal layer. Indeed, MMP-9 is co-secreted with MMP-9-lipocalin heterodimers,⁴⁶ which are specific for PMN secretion. Interestingly, whereas the lipocalin-MMP-9 complexes decrease in concentration progressively from the luminal to the abluminal layer, shorter bands corresponding to active MMP-9 and MMP-2 are released by the abluminal layer, providing evidence of a centrifugal gradient of MMP activation in the mural thrombus. MMP-8, a collagenase mainly se-

Figure 7. Stromal cell adhesion to fibrin is prevented by PMN medium, luminal extract, and elastase treatments. **A:** Phase contrast photomicrographs of BMSCs in culture. **A–C:** Like SMCs, BMSCs easily colonize the fibrin gel. Preincubation of the fibrin matrix with activated PMN secretates ($n = 5$), luminal extracts ($n = 5$), or purified elastase impaired BMSCs adhesion, spreading, and growth on the fibrin gel. Cell detachment was prevented by elastase inhibitors α_1 -antitrypsin and SLPI. *** $P < 0.001$, α_1 -antitrypsin and SLPI versus PMN medium, purified leukocyte elastase, and luminal thrombus extracts. **D and E:** Preincubation of the fibrin gel with PMN medium and luminal extract induces massive DNA fragmentation in marrow stromal cells indicating that anoikis had occurred. Addition of α_1 -antitrypsin and SLPI to PMN medium and luminal extracts before adsorption on fibrin prevented the retraction and DNA fragmentation of BMSCs. *** $P < 0.001$, α_1 -antitrypsin and SLPI versus PMN medium, purified leukocyte elastase, and luminal thrombus extract.

creted by PMNs,⁴⁷ showed a distribution similar to that of lipocalin-MMP-9.

MMP-9 and MMP-8 are present in the specific granules of PMNs,⁴⁷ whereas elastase is present in the azurophilic granules. Therefore, we hypothesized that PMN trapping and degranulation would also lead to the release of elastase within the thrombus. It has already been suggested by several studies that PMNs^{48,49} and leukocyte elastase^{50–52} could be involved in the development of abdominal aortic aneurysms. Recently, reduced plasma protease inhibitory activities have been reported in patients with abdominal aortic aneurysms.⁵³ Nevertheless, these studies focused on circulating blood markers⁵⁴ rather than on a role for leukocyte elastase released by PMNs trapped within the mural thrombus.

Immunohistochemistry demonstrated that elastase co-localizes with trapped PMNs and showed its ability to be retained, at least in part, in the fibrin polymer surrounding activated PMNs. It has been recently shown that elastase released by activated PMNs is able to degrade fibronectin^{23,39} and provoke SMC anoikis.^{23,39} In the present

study we showed the presence of solubilized, degraded fragments of fibronectin in the conditioned media of thrombi, providing further evidence of proteolytic activities within the thrombus. It has already been demonstrated that PMNs could induce detachment of endothelial cells from the extracellular matrix,^{55,56} as they do for smooth muscle cells.²³ It has been recently shown that serum-derived serine proteinase inhibitors are necessary for the spreading of SMCs on a fibrin gel⁵⁷ and prevented both fibronectin degradation and SMC apoptosis subsequent to the loss of anchorage.⁵⁸ However, as pericellular proteolysis is a necessary condition for cell migration, serine proteases with elastase activity could be also involved in SMC migration and proliferation.^{59–61} Thus, the role of elastase in mesenchymal cell physiology is probably dual, either participating in cell migration or inducing cell detachment and death, depending on the cellular source of the proteinase ie, SMCs,⁶¹ PMN leukocytes, or macrophages,⁶² and on the intensity and localization (clustering *versus* diffusion) of pericellular proteolysis.

Several proteases can bind to fibrin such as MMP-9,⁶³ plasmin, and plasminogen activators.⁶⁴ In the present study, only a small part of the elastase activity was detected in the neutral-conditioned medium of the luminal layer, providing evidence of little release of elastase by the fibrin matrix. The majority of the activity was retained within the thrombus. Immunohistochemistry not only localized elastase in the PMN granules, but also showed that it diffused locally into the fibrin surrounding PMNs and was retained there. To elute leukocyte elastase from fibrin, various buffers were tested with different ranges of molarity and pH. Finally, acetate buffer (pH 4.8) gave the best result, allowing us to elute elastase, preserving its activity after dialysis against PBS buffer.^{26,65}

We previously demonstrated that elastase-induced fibronectin degradation was associated with apoptosis of adherent SMCs²³ due to the loss of cell anchorage.⁶⁶ There is now much evidence that intact fibronectin is a substrate for cell adhesion⁶⁷ and can rescue adherent cells from apoptosis.¹⁵ Conversely, mutated binding domains of fibronectin induce fibroblast apoptosis.^{16,68} Similarly, adhesion to matrix is a necessary condition for growth and differentiation of BMSCs into mesenchymal cells capable of colonizing injured tissues and of differentiating into SMC-like cells.^{18,69} Our present data shows that extracts from the luminal layer of the mural thrombus are able to induce mesenchymal cell anoikis as are the products released during PMN activation; and that elastase inhibitors can prevent this loss of cell anchorage. That leukocyte elastase could be involved in the absence of tissue healing and cell re-colonization has already been suggested in leg ulcers.^{70,71} Therefore, we hypothesized that elastase released by PMNs and bound to the fibrin polymer could prevent cell adhesion and growth in the thrombus and thus inhibit its re-colonization. To demonstrate this point, we developed an *in vitro* approach of SMC and BMSC seeding, adhesion and growth on a fibrin matrix, preincubated or not with PMN secretion products or extracts of the luminal thrombus and elastase inhibitors. The elastase inhibitors used in this study are not entirely specific for elastase: α_1 -antitrypsin inhibits

leukocyte elastase, cathepsin G, and proteinase III,⁷² whereas SLPI inhibits both leukocyte elastase and cathepsin G.^{31,73} Thus, we cannot exclude the implication of cathepsin G in this phenomenon. However, several experiments were performed on fibrin matrices preincubated with PMN medium and cathepsin G activity was measured using a specific chromogenic substrate and no residual activity was detected on these fibrin matrices (data not shown). As recently shown,¹³ the present study confirmed that the fibrin polymer is an excellent matrix for adhesion and growth of SMCs and BMSCs, as well as for other adherent cell types,^{7–9} probably due to the presence of RGD motives. In contrast with the spontaneous adhesion and growth of SMCs and BMSCs on a fibrin matrix, the preincubation of this matrix with the supernatant of activated PMNs or with the extract of the luminal layer of the mural thrombus, followed by several washes, completely prevented cell anchorage and growth, leading to cell anoikis, as demonstrated by the apoptotic phenotype of non-adherent SMCs and DNA fragmentation in BMSCs. The fact that this loss of cell anchorage was prevented by addition of natural elastase inhibitors to conditioned media before incubation with the matrix, but not by several PBS buffer washes of the matrix after its preincubation with PMN-conditioned media, suggested that fibrin-bound elastase plays a predominant role in this phenomenon. In contrast, when natural elastase inhibitors were added after the preincubation of fibrin with PMN-conditioned media, they were unable to prevent mesenchymal cell anoikis, suggesting that fibrin-bound elastase is not sensitive to circulating inhibitors. Such a phenomenon has already been reported for other serine proteases such as plasmin and plasminogen activators⁷⁴ and for elastase bound to elastin.⁷⁵

In conclusion, our data first demonstrate that PMNs entrapped in the luminal pole of the mural thrombus of abdominal aortic aneurysms release proteinases such as MMP-9 and MMP-8 and elastase; and secondly, provide evidence that PMN elastase adsorbed in the fibrin matrix can prevent re-colonization of the thrombus by both SMCs and BMSCs. These data point to new therapeutic strategies involving the possible use of diffusible elastase inhibitors in the prevention of aneurysmal evolution via an enhancement of cellular healing by re-colonization of the mural thrombus.

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