Renewal of Mural Thrombus Releases Plasma Markers and Is Involved in Aortic Abdominal Aneurysm Evolution

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Human abdominal aortic aneurysm (AAA) expansion has been linked to the presence of a mural thrombus. Here we explored the mechanism of the continual luminal renewal of this thrombus and its ability to release biological markers potentially detectable in plasma. We also explored the ability of platelet inhibition to pacify the thrombus and to limit aneurysm progression in an experimental model. Blood samples and mural thrombi were collected in 20 AAA patients. In parallel, segments of sodium dodecyl sulfate-decellularized guinea pig aorta were xenografted onto the abdominal aorta of 30 rats to induce aneurysms. Fifteen rats received abciximab treatment and fifteen received irrelevant immunoglobulins. Procoagulant activity and platelet activation markers (microparticles, sP-selectin, sGPV, sCD40L) were increased threefold to fivefold in eluates from the luminal thrombus layer compared to other layers. All these markers were increased twofold to fivefold in patients' plasma compared to matched controls ($P < 0.005$). In the rat model, abciximab reduced both thrombus area and aneurysmal enlargement ($P < 0.05$). Platelet aggregation is probably responsible for the renewal of the thrombus in AAA. The luminal thrombus released markers of platelet activation that could easily be detected in plasma. Platelet inhibition limited aortic aneurysm expansion in a rat model, providing new therapeutic perspectives in the prevention

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Human acquired abdominal aortic aneurysms (AAAs)¹ are characterized by a progressive enlargement of the infra-renal abdominal aorta, spontaneously evolving toward rupture. This enlargement involves proteolytic degradation of the aortic media, adventitial inflammation and fibrosis, and the formation of a mural thrombus, which permanently interfaces circulating blood.² We^{3,4} and others^{5–7} have suggested that the mural thrombus, via its biological activity, could be one of the driving forces in AAA evolution characterized by abluminal fibrinolysis and compensated by luminal fibrinogenesis.

AAAs are characterized by both degradation of the extracellular matrix, mainly via activated matrix metalloproteinases (MMPs), and disappearance of smooth muscle cells.^{2,8} We recently showed that, at the abluminal pole of the aneurysmal mural thrombus, fibrin-bound plasminogen is converted to plasmin by activators present in the adjacent aneurysmal wall. This activation occurs at the interface between the wall-facing pole of the thrombus and the residual aneurysmal wall and subsequently leads to MMP activation, which may participate in aneurysmal enlargement.³ On the opposite side, the blood-facing pole of the mural thrombus, in contrast to the occlusive thrombus, maintains a permanent interface with the circulating blood components, resulting in its renewal. Therefore mural thrombi in AAA provide a unique opportunity to simultaneously study fibrin formation and degradation in the same sample. Experimental models have recently focused on the involvement of neutrophils in aneurysmal expansion.^{9,10} We and others observed that the mural thrombus trapped mainly neutrophils, which released MMP-9³ into the plasma^{11,12} and

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elastase into the fibrin network, subsequently impairing cellular healing.⁴

These data suggest that permanent luminal renewal of the mural thrombus could lead to the release of biological markers of thrombus activity into the plasma of patients and that pacification of this biological activity could represent a novel therapeutic target in the prevention of AAA evolution. Therefore, in the present study we explored the mechanism of luminal renewal of the mural thrombus in human AAAs. We showed that, in contrast with the intermediate and abluminal layers of the aneurysmal thrombus, the luminal part was greatly enriched in platelets, neutrophils, and their derived microparticles. Accumulation of activated platelets and phospholipids together with deposition of tissue factor (TF) resulted in a high thrombogenicity of the luminal pole of the thrombus, which was reflected by a high concentration of platelet activation markers in the plasma of AAA patients. Lastly, we demonstrated that abciximab, a platelet inhibitor that interferes with different integrins ($\alpha_{2b}\beta_3$, Mac-1, $\alpha_v\beta_3$), limited aneurysm development in an experimental rat model.

Materials and Methods

Study Participants

Twenty patients (male) aged 69 ± 8 years (mean $\pm$ SD; range, 61 to 76 years) with acquired AAA (diameter, ≥ 5 cm) were approached for study participation before surgery. Ethical committee advice (P030606) and patient informed consent were obtained (CCPPRB Paris-Cochin no. 2095). Blood was collected 24 hours before surgery on 0.129 mol/L sodium citrate from the 20 patients and from 20 sex- and age-matched healthy individuals. Cell-free plasma was obtained from blood by centrifugation for 15 minutes at $1550 \times g$ and then stored at -80°C .

Ex Vivo Study of Aneurysmal Thrombus

Mural thrombi collected during surgery were rapidly dissected into three layers: luminal, intermediate, and abluminal, as previously reported.⁴ The three thrombus layers were cut into small pieces (5 mm^3) and incubated in RPMI medium (Gibco, Invitrogen, Cergy Pontoise, France) for 24 hours at 37°C (2 ml/g of wet tissue). The conditioned media containing spontaneously eluted material (eluates) were then collected and stored at -80°C . Frozen samples were thawed and brought up to 37°C before assay performance.

For histological study, samples of the three layers of the mural thrombi were fixed in 3.7% paraformaldehyde, embedded in paraffin, and sectioned at 5 or 7 μm . The method of terminal dUTP nick-end labeling (TUNEL) was used to visualize DNA fragmentation (Roche Diagnostic, Meylan, France). 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. Cell nuclei were

shown by counterstaining with 100 ng/ml of 4',6'-diamidino-2-phenylindole hydrochloride.

Hematoxylin and eosin (H&E) was used to visualize cells and nuclei, orcein for the elastic fibers, and Masson's trichrome to visualize erythrocytes and fibrin within the thrombus. Biotinylated annexin V (Beckman Coulter, Roissy, France), which binds phosphatidylserine with high affinity and specificity,¹³ was used to probe for *in situ* anionic phospholipids with subsequent detection by horseradish peroxidase-conjugated avidin/biotin complexes and diaminobenzidine reaction. Platelets were identified using antibodies against GPIIIa (CD61, β_3) and P-selectin (CD62P) (DAKO, Glostrup, Denmark), and mesenchymal cells with an antibody against α -actin (DAKO). Immunostaining was amplified using the peroxidase-diaminobenzidine method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA).

Measurement of Microparticles

Microparticles, isolated from eluates or plasma as described by Biro and colleagues,¹⁴ were analyzed by flow cytometry (Coulter Epics XL with Expo 32 software, Beckman Coulter) using annexin V-FITC labeling of surface-expressed phosphatidylserine. In parallel, the cellular origin of the microparticles was determined using the following phycoerythrin-conjugated monoclonal antibodies: CD41, CD15, CD14, CD106, and glycophorin A (Immunotech, Beckman Coulter) for platelets, neutrophils, monocytes/macrophages, endothelial cells, and erythrocytes, respectively. To set the background fluorescence, we used annexin V in the absence of calcium or isotype irrelevant antibodies, as appropriate. Known amounts of fluorescent beads were added to the samples before analysis to allow calculation of absolute values of microparticles.

Coagulant Activity

The rate of fibrin generation (clotting time) was determined after addition of 100 μl of eluate or microparticle suspension to 100 μl of normal plasma using a one-step recalcification assay.¹⁵ A blocking antibody (10 $\mu\text{g/ml}$) against TF (American Diagnostica, Andrésey, France) as well as annexin V (1 $\mu\text{mol/L}$, Sigma, Lyon, France) were used to determine the contributions of TF and phosphatidylserine, respectively, to the shortening of clotting time. A prothrombinase assay¹⁶ and a specific assay for TF¹⁷ were used to quantitate phosphatidylserine and active TF in eluates and at the exposed surface of isolated microparticles.

Measurement of TF by Western Blot

Proteins eluted from the three layers of the thrombi were separated by 10% polyacrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with a primary antibody for TF (American Diagnostica). We used a hu-

Figure 1. Topography of platelet activation in the luminal layer of the thrombus. **A:** Macroscopic aspect of the mural thrombus showing the three layers: fresh red luminal layer, compact brown intermediate layer, and granular fibrinolytic abluminal layer. **B:** Microscopic aspect (Masson's trichrome, $n = 8$) showing fibrin (gray) and red blood cells (red) in the luminal layer, more compact fibrin polymer including broken-down red blood cells in the intermediate layer and the loose mesh of degraded fibrin in the abluminal layer. **C:** Cell nuclear staining by 4',6'-diamidino-2-phenylindole hydrochloride ($n = 6$). **D:** DNA fragmentation visualized by the terminal dUTP nick-end labeling method ($n = 6$). **E:** Immunostaining of GPIIIa (CD61, $\beta 3$) predominated in the luminal layer, reproducing the luminal patchy network: brown areas, **arrows** corresponding to GPIIIa/fibrin-rich areas, gray areas corresponding to red blood cell accumulation (**top**, $n = 4$). These areas were absent in the abluminal layer (**bottom**). **F:** Immunostaining of P-selectin (CD62P) also predominated in the luminal layer corresponding to GPIIIa/fibrin-rich areas (brown areas, **arrows**, **top**; $n = 4$). Trapped PMNs were co-localized with P-selectin in fibrin-rich areas (hematoxylin-stained nuclei, **bottom**). Original magnifications: $\times 100$ (**B–D**); $\times 400$ [**E**, **F** (**top**)]; $\times 1000$ (**F**, **bottom**).

man recombinant TF as positive control. Blots were developed by enhanced chemiluminescence.

Enzyme-Linked Immunosorbent Assay

Soluble glycoprotein V (sGPV) (Diagnostica Stago, Asnières, France), soluble P-selectin (sP-selectin), and soluble CD40 ligand (sCD40L) (R&D Systems, Lille, France) were measured in plasma samples and eluates by enzyme-linked immunosorbent assay according to the manufacturers' instructions. All assays were performed in duplicate.

Proof-of-Concept in Rats

To provide experimental evidence that platelet aggregation/activation plays a role in AAA evolution, experimental aortic aneurysms were induced by implantation of a segment (1.5 cm) of sodium dodecyl sulfate-decellularized guinea-pig aorta (xenogenic matrix) in 30 rat aortas,¹⁸ under pentobarbital anesthesia (authorization no. 75-214). As in human, this model is characterized by the presence of a thrombus,¹⁸ and *in situ* overexpression of PAI-1 and/or TIMP prevents aneurysmal expansion in this model.^{19,20} The initial diameter of the graft was measured *in situ*, without clamping, under a binocular microscope. Twenty-four hours later, rats were randomly allocated to two equal groups, treated for 6 weeks with either the Fab fragment abciximab (ReoPro, Lilly, Suresnes, France), an anti-integrin antibody able to inhibit platelet aggregation in rats²¹ (100 μ l per day of a 2-mg/ml solution, intraperitoneally), or irrelevant immunoglobulins (Sigma). At sacrifice, rats were deeply anesthetized, the graft diameter was measured again, and the aorta was sampled and fixed in formalin. Aortic samples were embedded in paraffin and cut. Sections were stained with Picrosirius red for collagen and fibrin, H&E for cells and nuclei, orcein for elastin, α -actin smooth muscle cell antibody (DAKO), and P-selectin antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). Thrombus areas were measured by morpho-

metric analysis on Picrosirius red slides using Histolab software (Microvision Instruments, Evry, France).

Statistical Analysis

Results for the study in humans are expressed as means $\pm$ SEM. Results were compared using a one-way analysis of variance for repeated measures. For the experimental rat study, we used the Wilcoxon and Mann-Whitney tests. Analysis of covariance was used for comparison of correlations. Differences between measured variables were considered significant for $P \leq 0.05$.

Results

Morphological Characterization of Aneurysmal Mural Thrombi

The macroscopic aspect of the mural thrombus enables one to easily define three layers: luminal, intermediate, and abluminal (Figure 1A). Microscopic observation of the red luminal blood-facing layer, in continuous contact with the flowing blood, showed a patchy distribution of areas in which either red blood cells (red) or fibrin (gray) predominate (Figure 1B). As previously described,⁵ cells, mainly neutrophils, predominated in the luminal layer of the thrombus,⁴ and many of these cells were undergoing apoptosis (Figure 1, C and D). There were no endothelial cells. Thrombus trapping of neutrophils predominated in fibrin-rich areas, associated with accumulation of platelets, as shown by immunostaining of GPIIIa and P-selectin (Figure 1, E and F). Frequently, neutrophils were surrounded by an empty circular space (Figure 1F, bottom), providing evidence of the ability of neutrophils to induce fibrinolysis and to phagocytose degraded fibrin as previously demonstrated.²² In the intermediate layer, intact red blood cells were absent and neutrophils rare, but the fibrin network remained dense. The brown abluminal wall-facing layer was acellular and the fibrin network was

Figure 2. Role of TF in fibrin generation. **A:** Time of fibrin generation of citrated plasma (clotting time) in presence of CaCl_2 and eluates from the different layers with (filled bar) and without (open bar) blocking TF antibody (RPMI used as control, $n = 18$). Time of fibrin formation was significantly shorter in luminal eluates as compared to those of intermediary and abluminal layers ($^{SS}P < 0.005$). For each layer, TF antibody increased significantly ($^{*}P < 0.05$) the clotting time by 18.5, 16.7, and 16.4%, respectively. This TF-dependent increase was not significantly different between the three layers. **B:** Moreover, a homogeneous distribution of TF throughout the thrombus layers was shown by Western blot (WB, $n = 6$).

degraded, providing evidence of abluminal fibrinolysis (Figure 1, B and C).⁵

Procoagulant Material Released by the Thrombus

Eluates from the different layers of the thrombus shortened the time of fibrin generation (clotting time) of a reference plasma from 270 seconds, when RPMI was used as a control, to 62 ± 6 (mean $\pm$ SEM), 120 ± 20 , and 110 ± 16 seconds for the luminal, intermediate, and abluminal layers, respectively (Figure 2A). The procoagulant activity was significantly greater ($P < 0.005$) for the luminal layer compared to the intermediate and abluminal layers.

The procoagulant material released by the thrombus consisted in part of TF, because a blocking TF antibody induced a significant ($P < 0.05$) prolongation of the clot-

Figure 3. Luminal thrombus released more phospholipids than other layers. **A:** Phosphatidylserine staining by biotinylated annexin V predominated in the luminal layer ($n = 4$). **B:** Chromogenic assays of TF (pg/g) and phosphatidylserine (pmol/L/g) procoagulant activities. There was no significant difference for TF in the three layers. In contrast, phosphatidylserine is more exposed in the luminal layer as compared to intermediate and abluminal layers ($P < 0.005$, $n = 12$). Original magnifications, $\times 200$.

ting times, regardless of the layer studied (Figure 2A). This partial inhibitory effect of the TF antibody was similar ($\sim 17\%$) for the three layers, consistent with the detection of similar amounts of TF protein by Western blotting and specific clotting assay (Figure 2B and Figure 3B) in the three layers. Therefore, the persistence of a predominant coagulation activity in the luminal layer with or without TF antibody ($P < 0.005$, Figure 2A), and the incomplete inhibitory effect of this antibody, suggested a gradient for other procoagulant factors.

Because exposure of phospholipids by platelet microvesicles is the main link between platelet activation and fibrin generation,^{23,24} we investigated phosphatidylserine localization in sections of thrombus stained with biotinylated annexin V. This revealed a predominant staining for phosphatidylserine in the blood-facing layer (Figure 3A), corresponding to the GPIIIa- and P-selectin-positive layer. The staining appeared to be mainly extracellular and granular and was negative in red blood cell-rich areas, although staining of neutrophils could not be excluded (Figure 3A). Chromogenic measurement of phosphatidylserine procoagulant activity on eluates also showed an increase ($P < 0.005$) of activity in luminal eluates (2.28 ± 1 pmol/g of throm-

Table 1. Release of Platelet Markers Predominated in the Luminal Part of the Thrombus Compared to Intermediate and Abluminal Layers ($n = 20$, $P < 0.005$; Top)

	Microparticles (nb/mg)	sP-selectin (ng/g)	sCD40L (pg/g)	sGPV (ng/g)
Thrombus				
Luminal	2248 ± 558**	2900 ± 524**	576 ± 120**	1028 ± 194**
Intermediate	664 ± 138	582 ± 90	140 ± 34	272 ± 46
Abluminal	464 ± 194	530 ± 86	138 ± 54	196 ± 44
Plasma	Microparticles (nb/μL)	sP-selectin (ng/ml)	sCD40L (pg/ml)	SGPV (ng/ml)
Patients	5906 ± 1163***	108.6 ± 9.8***	240.6 ± 29***	63.6 ± 7.5**
Controls	819 ± 136	40.8 ± 2.3	48.8 ± 4.5	39 ± 4.4

Markers were more elevated in plasma of patients with AAA ($n = 20$) compared to paired healthy controls ($n = 20$).
** $P < 0.005$ and *** $P < 0.0001$.

Figure 4. Blocking $\alpha_{2b}\beta_3$ promoted cell recolonization of the thrombus and limited aneurysm expansion in an experimental model of aneurysm in rats. **A:** Macroscopic aspects of experimental aneurysm (Sirius red staining) showed the important enlargement and thrombus (T) in controls ($n = 15$) as compared to abciximab-treated rats ($n = 15$) (less enlargement and small thrombus). **B:** P-selectin immunostaining predominated in the luminal thrombus layer of controls, whereas it was absent in abciximab-treated rats. **C:** H&E staining showed the luminal accumulation of PMNs in control thrombi (arrow) whereas abciximab limited this infiltration in the luminal layer of the thrombus. **D:** Elastic lamellae (arrows) were completely disorganized in thrombus-exposed media of controls whereas elastin was partly preserved in abciximab-treated rats. **E:** α -Actin-positive cells (arrows) were rarely present in the luminal layer of the thrombus in the control group. Moreover, these rare mesenchymatous cells did not spread on the fibrin (round morphology) and underwent apoptosis as suggested by the presence of pycnotic nuclei (top inset). In contrast, α -actin-positive cells appeared to spread and proliferate in the thrombus of abciximab-treated rats. Inhibition of platelet aggregation lead to limitation of thrombus area (F) and aneurysm diameter (G) in treated rats ($*P < 0.05$, $n = 15$). **H:** Lastly, there was a correlation between thrombus area and aneurysm dilatation in each group (controls, +; abciximab group, filled circle) that differed by the slope ($P < 0.05$). Original magnifications: $\times 2$ (A); $\times 400$ (B–E).

bus) compared to those of intermediate and abluminal thrombus (0.34 ± 0.18 and 0.22 ± 0.14 pmol/g, respectively) (Figure 3B). Moreover, analysis of eluates by flow cytometry revealed that the luminal thrombus layers released larger quantities of annexin V-positive microparticles (Table 1) compared to the intermediate and abluminal layers ($P < 0.0001$). The microparticles were essentially of platelet and neutrophil origin ($59 \pm 14\%$ and $36 \pm 12\%$, respectively), whereas none of monocyte/macrophage origin were detected. The presence of a greater number of microparticles probably accounts for the higher procoagulant activity of the luminal as compared to the other thrombus layers. Indeed, microparticles were shown to be the major procoagulant vehicles in the eluates: removal of the microparticles from the luminal layer eluates by centrifugation significantly prolonged clotting time (141 ± 14 seconds versus 62 ± 6 seconds, $P < 0.05$; data not shown).

Platelet and Coagulation Activation Markers in Eluates and Plasma

As shown in Table 1, markers of platelet activation (microparticles, sP-selectin, sCD40L, sGPV) were significantly increased in the eluates of the luminal layers compared to those from the intermediate and abluminal layers of the thrombi ($P < 0.01$). Therefore, we tested the hypothesis that these markers, released by thrombus activity, could be detected in plasma. Indeed, all these markers were significantly increased in the plasma from AAA patients compared to paired controls (Table 1, $P < 0.001$).

Effect of Abciximab on Aneurysm Development in Rats

Decellularized extracellular matrix xenografts were characterized by the development of aneurysms as previ-

ously described (Figure 4A).¹⁸ All aneurysms in the control group were associated with mural thrombi, which were grossly similar to what was observed in human thrombi, including platelet aggregates expressing P-selectin (Figure 4B), fibrin, and red blood cell areas, trapping of neutrophils (Figure 4C), medial elastin degradation (Figure 4D), and absence of mesenchymal cell colonization (Figure 4E). In particular, the model provides evidence of the impossibility of mesenchymal cells (α -actin-positive) (Figure 4E, top) to proliferate in the thrombus, as previously shown in humans.⁴ α -Actin-positive cells had pycnotic nuclei, suggesting that they were undergoing an apoptotic process (Figure 4E, top inset). In contrast, daily administration of abciximab for 6 weeks significantly reduced thrombus area (Figure 4F) and prevented, at least in part, the enlargement of the xenografted aorta in rats (Figure 4G). The beneficial effects of abciximab included decreasing P-selectin expression (Figure 4B), decreasing the binding of leukocytes to the blood-facing side of the thrombus (Figure 4C), limiting elastic fiber degradation in the media (thrombus-facing, Figure 4D), and increasing the number of smooth muscle cells adhering to the thrombus (Figure 4E). As compared to controls, α -actin-positive mesenchymal cells could spread and therefore proliferate within the luminal thrombus (Figure 4E, bottom inset). Lastly, there was a significant correlation between thrombus area and aneurysm diameter when all rats were considered ($R^2 = 0.65$, $P < 0.001$). However, intragroup analysis showed that abciximab treatment significantly reduced the slope (s) of the correlation as compared to the control group (abciximab: $s = 1.14 \pm 0.13$, $R^2 = 0.85$; control: $s = 2.70 \pm 0.57$, $R^2 = 0.67$; covariance analysis, $P < 0.001$) (Figure 4H), providing evidence of the anti-coagulant activity of abciximab but also showing that aneurysmal progression is in part independent of thrombus formation in this particular experimental model.

Discussion

As compared to occlusive thrombi, mural thrombi are characterized by a permanent dynamic interface with circulating blood components, responsible for continual renewal of the luminal part of the thrombus. There is now much evidence that the mural thrombus participates in the evolution of aneurysmal dilatation toward rupture in humans, whatever the localization and etiology: abdominal aorta,² thoracic aorta,^{25,26} and intracranial aneurysms,²⁷ as well as coronary aneurysms in Kawasaki disease.²⁸ The presence of a mural thrombus has been shown to be associated with a thinner arterial wall,²⁹ more extensive elastolysis, a lower density of smooth muscle cells in the media, and a higher level of immunoinflammation in the adventitia.⁶ This contrasts with a lower level of protease activities within the thrombus-exposed arterial wall as compared with the wall not exposed to thrombus activity.⁷ The mural thrombus is enriched with plasminogen-plasmin,³ and fibrinolysis predominates in the abluminal part of the thrombus.⁵ Acute signs of fibrinolysis (crescent sign on computed tomography scan)

have been associated with an immediate risk of rupture,³⁰ and plasma levels of plasmin-antiplasmin complexes are predictive of AAA evolution.¹² Plasma MMP-9, which probably originates from thrombus-entrapped neutrophils,³ is also a marker of AAA evolutivity.³¹ Therefore, the mural thrombus is a self-sustaining biological entity, renewal of which participates in aneurysmal evolution.

Morphologically, the thrombus is composed of a fresh luminal layer, where its renewal occurs, contrasting with the acellular abluminal layer, which is the site of thrombus degradation. Only the luminal layer contains important numbers of cells,⁵ mainly neutrophils,⁴ that predominate in the fibrin-rich areas. The present study shows that the luminal layer is the site of platelet accumulation and activation as well as fibrin co-localization, in accordance with previous reports.³² The observed co-localization of neutrophils, platelets, and fibrin most probably results from both leukocyte capture on adherent activated platelets and cell interactions with fibrin.³² The fate of neutrophil accumulation after thrombus formation was documented long ago.³³ Moreover, neutrophils could initiate fibrinolysis because elastase can cleave fibrin²² and neutrophils can phagocytose products of fibrin degradation.³⁴ The observation of an empty circular space surrounding neutrophils probably provides evidence of this fibrinolytic activity. The cell-cell contacts are mediated by interactions between neutrophil PSGL-1 and CD15 with platelet P-selectin, which is rapidly mobilized from secretory granules to the platelet surface on stimulation by thrombin or other mediators.³² Fibrin could serve as a bridging molecule between platelet $\alpha_{2b}\beta_3$ and neutrophil β_2 -integrins and L-selectin.^{35,36} Binding of Mac-1 (CD11b/CD18, $\alpha_M\beta_2$), mobilized from the intracellular pool in neutrophils,³⁷ to fibrin/fibrinogen³⁸ also participates in leukocyte adhesion³⁹ to sites of fibrin deposition and coordinates the activation of factor X.⁴⁰ As recently demonstrated, erythrocyte aggregates could also participate in platelet activation by releasing important quantities of ADP/ATP.⁴¹

In parallel, we showed that the luminal layer of the thrombus is characterized by high levels of soluble markers of platelet activation (sP-selectin, sCD40L, sGPV), extensive neutrophil apoptosis, large numbers of microparticles mostly derived from platelets and neutrophils, and the presence of active TF. The ability of neutrophils to express TF is controversial, but evidence has been provided that circulating platelets⁴² or neutrophils⁴³ may capture TF-bearing microparticles derived from monocytes before being incorporated within a thrombus.⁴⁴ In these conditions, *in situ* production of TF-bearing microparticles could have been promoted by local platelet P-selectin membrane expression and shedding,⁴⁵ as well as by sCD40L release.⁴⁶ Alternatively, blood-borne TF could have been taken up by the growing thrombus, as demonstrated previously *in vitro* in a flow chamber⁴⁷ and *in vivo* in a mouse model of thrombosis⁴⁸ and derived from encrypted sources in blood through a P-selectin/PSGL-1-mediated mechanism.^{44,49} TF may also have been transferred from apoptotic cells because microparticles behave as diffusible vectors responsible for transcellular exchange of biological materials.⁵⁰ Micropar-

ticles exhibit negatively charged phospholipids, chiefly phosphatidylserine, at their surface, accounting for part of their procoagulant character.²⁴ At a constant TF concentration, the phosphatidylserine content is the rate limiting factor for the assembly of tenase and prothrombinase complexes and thrombin generation, which accounts for the greater thrombogenicity of the luminal layer of the thrombus observed.²³

In a second step, we showed a significant increase in platelet activation markers in the plasma of cases with AAA compared to paired controls. That microparticles,⁵¹ sP-selectin,⁵² sCD40L,⁵³ and sGPV⁵¹ are all potential plasma markers in atherothrombosis has already been proposed through clinical studies in patients with acute coronary syndromes or strokes. However, the direct relation of these plasma markers to mural thrombus activity has never been established, and these markers have never been explored and used as surrogate markers in AAA. Exploration of the prognostic value of these plasma markers in AAA evolution, enlargement, occurrence of endoleaks, and risk of rupture requires further prospective studies in patients with small aneurysms or with AAAs treated by endovascular graft.¹

In a last step, as proof of the concept, we tested the role of thrombus activity in aneurysm evolution by exploring the hypothesis that inhibition of platelet aggregation might pacify the thrombus and prevent aneurysmal enlargement in an experimental model. We chose abciximab because $\alpha_{2b}\beta_3$ (GPIIb/IIIa) and $\alpha_v\beta_3$ integrins are the main factors involved in platelet fibrinogen/fibrin and platelet/cell interactions and this blocking antibody is efficient in reducing microparticle release, P-selectin expression and release, and platelet procoagulant activity.^{54,55} We chose the model of the decellularized xenograft in rats¹⁸ because the initial cause of aneurysm formation is direct immune injury to the xenogenic matrix that is totally independent of the coagulation cascade. Nevertheless, the model is characterized by the early development of a mural thrombus, which could contribute to aneurysm evolution as in humans. The mural thrombus in the experimental model presented numerous similarities with human aneurysmal thrombi. With abciximab, we observed a clear and significant benefit on aneurysm pathophysiology. The aneurysmal diameter was significantly reduced and the elastin network more well preserved in the arterial wall of the abciximab-treated group than in controls. Interestingly, the thrombus is reduced in size and, to some degree, pacified, showing less expression of P-selectin, less recruitment of neutrophils, and more colonization by mesenchymal cells. As already demonstrated by cell therapy approaches in this model, smooth muscle cell recolonization is able to prevent and reverse the effects of proteolytic injury responsible for graft enlargement and rupture.¹⁹ Abciximab treatment partially reproduces this beneficial effect in the same model. Abciximab is not highly specific of $\alpha_{2b}\beta_3$ as it presents equivalent affinity and functional blockade of $\alpha_v\beta_3$ integrins⁵⁴ and the leukocyte integrin Mac-1 ($\alpha_M\beta_2$).⁵⁶ Potential interactions of abciximab with other integrins shared by

inflammatory cells (ie, monocytes and neutrophils),³⁷ vascular smooth muscle cells, red blood cells, endothelial cells, and platelets may enhance or limit the beneficial effect of platelet inhibition in our model. Indeed, the redistribution of abciximab between different integrins shared by platelets and leukocytes may provide prolonged efficacy.⁵⁴ Our observation that abciximab decreased the slope of the linear relationship between aneurysm diameter and thrombus area suggests that the efficacy of abciximab on AAA enlargement was primarily because of its preventive effect on thrombus formation, whatever the molecular complexity of these interactions. Nevertheless, further studies exploring separately and more specifically the different components of platelet/cell interactions (ie, $\alpha_{2b}\beta_3$, $\alpha_v\beta_3$, and Mac-1) should be performed in the near future in our model, before proposing such a therapeutic strategy in prevention of AAA progression in human.

In conclusion, this study is the first to explore the mechanism of mural thrombus renewal in human AAA. We observed that the blood-facing (luminal) layer of the thrombus has more procoagulant activity than the intermediate and the abluminal layers, which could be related to platelet activation, aggregation, and the generation of exposed phosphatidylserines. Therefore luminal platelet activation could release procoagulant mediators that could be detected as biological markers in plasma of AAA patients, providing evidence of thrombus activity. Moreover, thrombus pacification limited aneurysmal enlargement in an experimental model. These data provide new perspectives in therapeutic strategy for preventing aneurysm expansion in humans.

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