Annexin V specifically binds with nanomolar affinity to PS, which is exposed to the surface of activated platelets\(^\dagger\) and apoptotic cells.\(^\dagger\) Therefore, radiolabeled \(^{99m}\)Tc-annexin-V (ANX) has been previously used for in vivo scintigraphic imaging of both apoptotic cells in animals and humans,\(16\)–\(19\) and acute platelet-rich thrombi in animals.\(12\),\(20\) Nevertheless, the ability of ANX to assess renewal activity in chronic mural thrombus, at the interface between circulating blood and mural thrombus, has not yet been reported. Because PS exposure is one of the mediators of the platelet activation–induced fibrin formation, ANX imaging could provide noninvasive functional information on the renewal rate of mural thrombus, which maintains a permanent interface with flowing blood. The present study aimed to evaluate the ability of ANX imaging to assess activity of the mural thrombus in an experimental model of aneurysm in rats.\(^\dagger\) Moreover, the clinical applicability was further tested on human samples of excised mural thrombus of AAA harvested from patients undergoing surgery.

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**Key Words:** platelets • aneurysms • vascular biology • phosphatidylserine

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**Background**—The mural thrombus of abdominal aortic aneurysms (AAA) is involved in aneurysm progression via several interdependent biological processes including platelet activation. \(^{99m}\)Tc-annexin V (ANX) is a scintigraphic tracer that binds to phosphatidylserine exposed on activated platelets and apoptotic cells. Here, we evaluated the potential of ANX imaging to assess mural thrombus biological activity in an experimental AAA model. The clinical applicability was further tested ex vivo on human samples of excised AAA thrombi.

**Methods and Results**—Experimental AAA was created by infusing elastase into infrarenal abdominal aorta in 17 rats, and 6 sham-operated rats were used as controls. Abdominal ANX scintigraphy was performed 2 weeks later followed by quantitative autoradiography and histological studies. Among the 13 rats which developed AAA, 11 displayed intense ANX uptake within AAA by scintigraphy. ANX uptake in the aneurysms on planar and single-photon emission computed tomography (SPECT) imaging was higher than that observed in infrarenal aorta of sham-operated controls (target/background ratio: 5.7±0.9 versus 1.33±0.21; P<0.005 for SPECT). Aneurysm-to-background activity ratios obtained by scintigraphy correlated with ANX activity in corresponding autoradiograms (R=0.69; P<0.02). This activity was located in the thrombus area where activated platelets and polymorphonuclear leukocytes accumulated. Similar patterns were also found in all of the 7 human AAA thrombi harvested during surgery.

**Conclusions**—ANX imaging may assess mural thrombus renewal activity linked to permanent flowing blood interface.

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Annexin V specifically binds with nanomolar affinity to PS, which is exposed to the surface of activated platelets\(^\dagger\) and apoptotic cells.\(^\dagger\) Therefore, radiolabeled \(^{99m}\)Tc-annexin-V (ANX) has been previously used for in vivo scintigraphic imaging of both apoptotic cells in animals and humans,\(16\)–\(19\) and acute platelet-rich thrombi in animals.\(12\),\(20\) Nevertheless, the ability of ANX to assess renewal activity in chronic mural thrombus, at the interface between circulating blood and mural thrombus, has not yet been reported. Because PS exposure is one of the mediators of the platelet activation–induced fibrin formation, ANX imaging could provide noninvasive functional information on the renewal rate of mural thrombus, which maintains a permanent interface with flowing blood. The present study aimed to evaluate the ability of ANX imaging to assess activity of the mural thrombus in an experimental model of aneurysm in rats.\(^\dagger\) Moreover, the clinical applicability was further tested on human samples of excised mural thrombus of AAA harvested from patients undergoing surgery.

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Methods

In Vivo Assessment of ANX Uptake in a Rat Model of AAA

Rat Model

Male Lewis rats (n = 17) aged 12 weeks were purchased from CEJ, Le Genest, France. AAA were induced by infusion of elastase according to the previously described method. Under pentobarbital anesthesia (4 mg/100 g body weight [bw]; Ceva Santé Animale), and using a dissecting microscope, about 15 mm of the infrarenal aorta was separated from the vena cava and collateral vessels were ligated when necessary. Subsequently, a catheter was introduced into the left femoral artery and advanced up into the infrarenal aorta. The aorta was clamped below the renal arteries, and at about 10 mm a distal thread was tightened around the catheter, performing a closed perfusion chamber. Three units of pancreatic porcine elastase (E-1250, Sigma) in 800 \( \mu \)L NaCl 9\(^{9/0}\) were infused transmurally during 1 hour, using a pressure perfusion pump. The segment was then rinsed, flow re-established and surgical wounds closed. Six sham-operated rats were obtained by a similar procedure in the absence of elastase.

The procedure and the animal care complied with the principles of animal care formulated by the National Society for Medical Research. This study was performed under the authorization No. 75-101 of the French Ministry of Agriculture.

Study Design

Two weeks after elastase infusion, rats were injected with ANX and abdominal scintigraphic imaging was performed. Then rats were euthanized for quantitative autoradiography and immunohistochemical studies of the performed aortic segment.

ANX-Labeling Procedure

ANX was prepared by injecting sodium pertechnetate (400±20 Tc-pertechnetate [MBq] drawn-up from a \(^{99m}\)Tc generator, freshly eluted) with stannous tricine in a sterile vial containing annexin V (Theus Imaging Corp). After shaking, the preparation was left to stand 15 minutes at room temperature. The quality control was performed with instant thin-layer chromatography, using citric acid/dextrose solution as eluant. The radiolabeling yield was always superior to 88%.

ANX Scintigraphy

Scintigraphic imaging was performed under pentobarbital anesthesia (4 mg/100 g bw; Ceva Santé Animale) in all 17 rats, after intravenous injection of ANX.

In 14 rats, planar anterior abdominal images were obtained 0 to 15 minutes (dynamic acquisition: 15 images, image duration: 60 seconds), 30 minutes, 45 minutes, 1 hour and 2 hours (static acquisitions of 10 minutes duration) after ANX injection. They were performed once after injected dose of 37 MBq in 6 animals, twice after injected doses of 37 MBq then 74 MBq at 3 days interval in 3 animals, and once after injected dose of 74 MBq in the other 5 animals. In addition, 6 rats (3 of which had previously undergone planar imaging) underwent abdominal X/tomographic imaging (single-photon emission computed tomography [SPECT]) acquisition after 74 MBq ANX injection: mod-list tomographic acquisition was performed during continuous rotation of the animal placed between 2 parallel collimators (360° rotation per minute, acquisition duration: 60 minutes from 1 hour to 2 hours after ANX injection). All acquisitions were performed using a dedicated small animal XIMAGER-S/CT system (Biospace Mesures) equipped with parallel low-energy high-resolution collimators (matrix 128×128, 15% energy window centered on 140 KeV). ANX uptake in AAA areas was visually assessed, and activity (mean counts per pixel) ratios between aneurysm areas (determined on early dynamic images) and underlying background areas were computed on planar images, as well as on transversal tomographic images.

ANX planar and tomographic imaging was also performed in 6 sham-operated controls injected with 74 MBq of ANX.

Autoradiography

All rats were euthanized 3 to 4 hours after intravenous injection of ANX. A ring of the thoracic and the infrarenal aortas were dissected out, frozen and cut into transverse sections of 20-\( \mu \)m thickness, which were exposed in a radioimager (Instant Imager; Packard) for 16 hours. The activity (counts/mm\(^2\) and total counts) was determined on autoradiograms from aneurysm and normal thoracic aorta sections. According to calibration studies previously reported, with activity standards of tissue-equivalent homogenates, 50 counts/mm\(^2\) of ANX approximated 210 kBq/mg in autoradiography.

Histological Studies

Sections used for autoradiography were stained with hematoxylin-eosin and picro-indigocarmin or toluidine blue (1%) to determine sites of tracer uptake within aneurysmal structures. To localize apoptotic cells, the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL; terminal transferase-mediated dUTP nick end-labeling) method\(^{23}\) was used to visualize fragmented DNA on frozen sections (7-\( \mu \)m thickness) according to the manufacturer’s instructions (Roche). A positive control with DNase I (Qiogene) treatment (3 U/mL) and a negative control omitting the use of terminal transferase were simultaneously performed. Immunohistological studies were performed on frozen AAA sections fixed in acetone. After inactivation of endogenous peroxidase by 0.3% H\(_2\)O\(_2\) for 20 minutes, blockade of endogenous biotin sites (Dako) and of nonspecific sites for irrelevant immunoglobulin (Dako, negative controls), incubation with one of the following primary specific antibodies was performed for about 1 hour at room temperature. Goat anti-mouse P-selectin antibody (SC 6943, Santa Cruz Biotechnology; Santa Cruz, Calif, USA; 4 \( \mu \)g/mL) was used to localize activated platelets, polycyonal rabbit anti-rat PMN antibody (CLAD 51140, CEDARLANE; Hornby, Canada; dilution 1/3000) and monoclonal mouse anti-rat CD68 to identify macrophages (ED1, MCA341R; Serotec, Oxford, UK; 0.2 \( \mu \)g/mL). The secondary antibodies used were, respectively, anti-goat (Jackson Laboratory, Inc, Bar Harbor, Me) and anti-rabbit (Life Sciences, Basel, Switzerland) IgG, both coupled with peroxidase and rat-adsorbed biotinylated anti-mouse IgG (Vector Laboratory, Burlingame, Calif). For this latter, an incubation with peroxidase coupled with streptavidine (Dako) was done before to reveal peroxidase activity with diamobenzidine as substrate.

ANX Uptake and Histological Studies in Excised Human AAA Samples

Aneurysm thrombi (5-mm thickness) obtained from 7 patients undergoing surgical AAA resection were incubated with either ANX (2 MBq/mL), or \(^{99m}\)Tc-labeled serum albumin (Vasculocilis, Shering, 2 MBq/mL) in RPMI 1640 medium for 30 minutes at room temperature. Additionally, one thrombus was incubated with ANX both in the presence and 10\(^{\pm}\) of EDTA (Ca\(^{2+}\)-complexing agent). After incubation, the thrombus slices were rinsed 5 times with ice-cold RPMI 1640 medium. They were placed on the \( \gamma \)-camera detector (Biospace Mesures) for scintigraphic 45 minutes static acquisition. Then after freezing, they were cut into 20-\( \mu \)m transverse sections for quantitative autoradiography and into 10-\( \mu \)m sections for further immunohistological studies. Activity ratios between the luminal and the basal parts of the thromb (delineated according to macroscopic features) were calculated on scintigrams and autoradiograms.

The immunohistological detection of platelets was performed using mouse anti-human GPIIIa (M-0753, Dako, Glostrup, Denmark; 2 \( \mu \)g/mL) and detection of activated platelets using mouse anti-human P-selectin (M-7199, Dako) and anti-human CD-66b (Dako) antibodies. Detection of PMN and macrophages was performed using, respectively, mouse anti-human CD-66b (Dako) and anti-human CD-68 (Dako) antibodies. Incubation of primary antibodies was performed overnight at 4°C after inactivation of endogenous peroxidase with 0.3% H\(_2\)O\(_2\) and blocking nonspecific sites for primary antibody with 2% horse serum. A biotinylated universal secondary antibody raised
in the horse and the ABC complex (Vectastain universal ABC kit: PK 6200; Vector Laboratory) were used and peroxidase was revealed by DAB-chromogen (Dako, K-3466).

Statistical Analysis

Data are expressed as means±SEM. The unpaired t test was used to compare target-to-background activity ratios on ANX scintigraphic images in rats with and without aneurysm. The paired t test was used to compare ANX activity (radioactive counts) measured on autoradiograms in aneurysm and normal thoracic aorta sections, as well as to compare ANX activity in luminal versus basal layer of human aneurysm thrombus on scintigraphy and autoradiography. Linear regression curves and Pearson correlation coefficients were obtained by the least-squares methods to relate ANX aneurysm-to-background activity ratios obtained by scintigraphy and total ANX activity in corresponding aneurysm sections obtained by autoradiography. The level of significance was set at P<0.05.

Results

Rat AAA Experiments

Two weeks after elastase perfusion, 13 rats developed AAA and 4 did not. The aneurysmal size ranged from about 5 to 17 mm in diameter and 7 to 18 mm in length.

Visual analysis of scintigraphic images revealed increased ANX uptake in AAA (“positive scintiscan”) of 11 of 13 rats (84.6%, 95% CI [65;100]), on both planar and tomographic scans compared with control unperfused rats (Figures 1 and 2), whereas 2 of 13 rats with AAA showed negative images. Tracer uptake in scintiscan-positive aneurysms was more clearly visualized when the injected dose was high (74 MBq). Aneurysm-to-background ANX activity ratios obtained in the rats with AAA were significantly higher than the infrarenal aorta-to-background ratios obtained in the elastase-perfused rats with no AAA, and significantly higher than the infrarenal aorta-to-background ratios obtained in sham-operated controls (Figure 3). Autoradiography showed higher ANX activity in aneurysms compared with control thoracic aortic sections (Figure 1): 347±157 versus 19±9 counts/mm², P<10⁻⁶, with mean aneurysm-to-normal aorta ratio of 26±13 for the rats with positive scintigraphy, and of 6±2 for the 2 rats with AAA and negative scintigraphy. The ANX activity ratios calculated on the scintiscans (except those obtained 0 to 15 minutes after injected dose of 74 MBq) correlated with total ANX counts in aneurysmal sections obtained by autoradiography (coefficient correlation =0.69, P<0.02, standard error for the regression: 0.26, for linear regression of activity ratios obtained by 1H-scintiscans on activity obtained by autoradiography, see Figure 4). Histologically, these rat aneurysms were characterized by extensive loss of elastic fibers, infiltration of inflammatory cells and the presence of an intraluminal thrombus in all cases. Analysis of sections used for autoradiography showed that ANX uptake localized mainly in the luminal part of the thrombus where activated platelets (expressing P-selectin) and leukocytes accumulated (Figure 5). In this luminal layer, the presence of numerous PMN and the more scarcity of macrophages was confirmed by immunohistochemistry (Figure 5F and 5G). Macrophages were mainly localized within the adventitia. In some cases, parts of the luminal thrombus did not show any ANX-labeling or leukocyte accumulation. In the 2 rats with negative scintigraphy and very faint ANX activity on autoradiography, AAA were of a small size (5 to 6 mm of diameter), with a tiny thrombus occupying a very small part of the aneurysmal circumference in one case, and showing very little fibrin accumulation in the other case. TUNEL-labeling showed that fluorescent nuclei were not located in the most...
luminal part of the thrombus, containing PMNs with nuclei of normal appearance which probably correspond to freshly recruited cells, but more deeply where numerous cell nuclei appeared to be abnormally condensed.

Rats which did not develop aneurysms (n=4) and whose aortic diameter was 3 to 4 mm, did not show any significant ANX uptake in the elastase-perfused infrarenal aorta by scintigraphy. In 3 of them, autoradiography was also negative and no thrombus was detected by histological analysis in elastase-perfused aorta. However, in one rat, autoradiography revealed increased tracer activity in the perfused aortic segment compared with the normal thoracic aorta taken as control (380 versus 9 counts/mm²). This radioactivity was localized within a small thrombus containing numerous PMNs.

Human AAA Experiments

The human aneurysmal thrombi obtained after AAA surgical resection were 2 to 6 cm in diameter. Macroscopically, all thrombi displayed 2 distinct layers: a red luminal layer, which is in contact with the circulating blood in vivo, and a brownish basal layer in contact with the aortic wall. Scintigraphic as well as autoradiographic images of the thrombi incubated with ANX revealed, in all cases, significantly increased ANX activity in the luminal compared with the basal layer: 83±36 versus 37±19 counts per mm² (P<0.005) on scintigraphy, and 344±96 versus 41±16 counts per mm² (P<0.005) on autoradiography (Figure 6). On the other hand, no significant difference in the nonspecific BSA activity was observed between the 2 layers on scintigraphic images: 25±12 versus 22±5 counts per mm². In addition, ANX binding within the luminal thrombus layer was abolished when incubation was performed in presence of the Ca²⁺ complexing agent (EDTA). Also in 2 cases, increased ANX activity was observed along macroscopically visible cracks in the basal layer of the thrombus (Figure 6).

The histological and immunohistochemical study of human thrombi sections showed that ANX activity was localized within the luminal part of the thrombus where PMNs and activated platelets (P-selectin positive) accumulated (Figure 6). These data were confirmed by the observed high density of PMNs (CD-66b positive cells) in the luminal layer of human thrombus contrasting with rare macrophages (CD-68 positive cells; Figure 6).

Discussion

In the rat experimental model of an elastase-induced infrarenal aortic aneurysm, intense radiolabeled annexin-V uptake was detected in vivo after intravenous injection on both scintigraphic images and autoradiograms in the luminal part of the mural thrombus, which is the site of thrombus renewal activity involving activated platelets and PMNs trapping. A
significant correlation was obtained between ANX uptake ratios on imaging and ANX activity in aneurysm sections on quantitative autoradiography. A similar pattern of increased specific ANX binding in the biologically active luminal layer was observed ex vivo in excised mural thrombi of human AAAs.

**ANX Uptake in the Luminal Part of the Aneurysmal Thrombus**

AAAs are usually characterized by the presence of a nonocclusive thrombus, through which blood continues to flow. This thrombus thus maintains the interface with circulating blood at the luminal pole, and with the aneurysmal wall at the abluminal pole. The mural thrombus is a complex laminated structure with several thick layers of fibrin and a most recently formed red luminal layer. This luminal part of the mural thrombus is composed of patchy areas of red blood cells and fibrin, trapped PMNs, aggregated platelets and plasma components. The oldest abluminal part of the thrombus is composed of a loose network of degraded fibrin, in which initial components, including red blood cells, cannot be identified, whereas dying neutrophils are present.

During activation, aggregation and disintegration, platelets expose aminophospholipids, mainly phosphatidylserine, which is stainable by annexin V. In our experimental rat model of AAA, after in vivo injection of ANX, a marked accumulation of radioactivity was observed in the luminal part of the thrombus by autoradiography. The same observation was made in excised human aneurysms, after incubation with ANX ex vivo. Furthermore, we have recently found a greater release of annexin V/fluorescein isothiocyanate–labeled microparticles of platelet and neutrophil origin from the luminal part of human aneurysmal thrombi, associated with a higher procoagulant activity, compared with the intermediate or abluminal parts. In the present study, histological observations were comparable in human and rat thrombi, showing the enrichment of the most luminal part with platelet markers (anti-GPIIIa, anti–P-selectin) and polymorphonuclear leukocytes. The importance of PMNs in thrombus evolution has long been known. During fibrin formation, PMNs are trapped then accumulate in the thrombus (12-fold more numerous in clots compared with circulating blood), because they have a high affinity for the fibrin-fibronectin network and bind to platelet-exposed P-selectin via the expression of its selective polysaccharide ligand. Recent data show that PMNs trapping and elastase release are linked to platelet activation. In the present study, TUNEL-staining of PMNs was mainly positive in the intermediate and not the most luminal zone of the thrombus, suggesting that the observed ANX uptake in this latter area was mostly related to binding to platelet-exposed phospholipids.

Because ANX binds to exposed phosphatidylserines whatever their origin, ANX-imaging can be positive in the presence of either platelet activation or cell apoptosis. A high uptake of radiolabeled annexin V was previously demonstrated in rabbit and swine models of fully occlusive thrombi formed 1 to 2 hours before annexin-V intravenous injection, as well as in acute porcine left atrial thrombi formed by crush injury. In this latter early acute study, ANX visualized the whole thrombus probably because platelet activation was present in the whole recent thrombus (2 hours) biologically corresponding to the most luminal renewal part of the chronic mural thrombus in AAA. In contrast, in ballooning-induced atherosclerotic plaques in hypercholesterolemic rabbits, Kolodgie et al reported significant ANX accumulation mainly associated with the presence of apoptotic macrophages. This discrepancy may be attributable to differences in the experimental models, and to the fact that the presence of aggregated platelets and phospholipid-dependent fibrin formation was not explored in their study.

**In Vivo Imaging of Luminal Thrombus in Experimental Rat AAA With ANX Scintigraphy**

ANX allows sensitive in vivo scintigraphic detection of apoptotic cells in various animal models, including chemoradiotherapy-induced tumor cell death, organ transplant rejection, acute and subacute inflammation, ischemia/reperfusion, and atherosclerosis. In humans, ANX has been successfully used for scintigraphic detection of cardiac transplant rejection, myocardial infarction, early humoral response to chemotherapy, and unstable atherosclerotic plaques. Also, ANX allowed scintigraphic detection of acute porcine left atrial thrombi formed by crush injury, with a mean left atrial appendage/blood ratio on tomographic images at 2 hours of 3.90±1.12 ratios in animals with a thrombus compared with 1.01±0.23 ratios in open-chest controls (P<0.001). In the present study, all experimental aneurysms with positive autoradiography were clearly visualized by scintigraphy, both on planar and SPECT images. The lower signal-to-background ratios on anterior planar images than on SPECT images (5.7±0.9 ratio) are explained by a higher background activity related to mild ANX uptake in vertebrae superimposed on the aorta. In contrast to planar imaging, SPECT allowed clear discrimination between ANX activity in aneurysm and in vertebrae, as seen on Figure 1. Planar ANX scintigraphy was negative in 1 rat with no aneurysm but high ANX activity observed by autoradiography within a small thrombus containing numerous PMNs. It may be assumed that SPECT would have been positive in this case, because of the better signal-to-background ratio. ANX uptake in aneurysmal areas by imaging was correlated with total ANX activity in aneurysm sections by autoradiography. Therefore, ANX-imaging may constitute a noninvasive in vivo method providing functional information on mural thrombus activity in abdominal aortic aneurysms.

**Potential Clinical Interest of Abdominal Aortic Aneurysm ANX Scintigraphy**

Surgical management is recommended for aneurysm diameter superior to 5.5 cm. On the other hand, systematic screening for AAA in old men reduced the frequency of acute operations and specific mortality. The development of functional imaging may be appropriate for evaluating aneurysm evolution, which is linked to biological processes involving inflammation and mural thrombus renewal. A previous study using 18-FDG positron emission tomography suggested a possible association between increased metabolic...
activity on imaging, mainly attributable to inflammatory cells, and AAA expansion and rupture. However, the specific role of the mural thrombus in aneurysmal evolution toward rupture has been pointed out in acquired AAA and in other aneurysmal pathologies in humans. In this respect, toward rupture has been pointed out in acquired AAA and in specific role of the mural thrombus in aneurysmal evolution None.

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Disclosures
None.

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