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Abstract

Pathogenesis of the abdominal aortic aneurysm has been attributed to neovascularization of the aortic wall. However, it is not clear whether angiogenesis persists in the aneurysm. In sections of aneurysms, we determined the immunohistochemical distributions of the \( \alpha_v \beta_3 \) integrin, tenascin and endothelial nitric oxide synthase (eNOS), which are markers respectively, of angiogenesis, matrix remodeling and vasoregulatory function. In addition, we used reverse transcription followed by in situ PCR, to determine the distribution of \( \alpha_v \) mRNA. All aneurysm specimens exhibited extensive increases of wall vascularization as compared with the control aortic wall, and showed the presence of perivascular inflammatory exudates containing macrophages and lymphocytes. The neovascularization consisted of thick-walled vessels in the media and adventitia, and capillaries in the subintima. The majority of vessels stained positively for the \( \alpha_v \beta_3 \) antigen and eNOS. Tenascin was deposited as bands that circumscribed thick-walled vessels. The distribution of \( \alpha_v \) mRNA was extensive and was positive even in those vessels that failed to stain for the \( \alpha_v \beta_3 \) protein. No staining was evident in control aortas for the \( \alpha \beta_3 \) antigen, tenascin or \( \alpha_v \) mRNA. The upregulation of \( \alpha_v \) mRNA and the \( \alpha_v \beta_3 \) integrin in blood vessels surrounded by a matrix expressing tenascin, indicates that angiogenesis is an ongoing process in the mature aortic aneurysm.

Keywords: angiogenesis; aortic aneurysm; extracellular matrix; immunohistochemistry; integrin; RT in situ PCR

Introduction

The pathophysiology of the abdominal aortic aneurysm (AAA), which carries an annual mortality in the USA of \( \sim 15,000 \) (Brophy et al., 1991) is not well understood; and its treatment is restricted to surgical repair with its attendant risks of morbidity and mortality. The pathophysiological understanding of the aneurysm is based largely on biochemical and immunohistochemical studies that indicate aortic wall remodeling and proteolysis of matrix proteins (Tilson et al., 1990). A multicellular inflammatory infiltrate and increased expression of tissue-type and urokinase-type plasminogen activators have also been demonstrated in the aneurysm wall (Koch et al., 1990; Brophy et al., 1991; Schneiderman et al., 1995).

A conspicuous feature of the aneurysm is neovascularization of the aortic wall, which, in contrast to normal, becomes enriched with microvessels (Koch et al., 1990; Tilson et al., 1990; Brophy et al., 1991; Holmes et al., 1995; Schneiderman et al., 1995). Although the significance of the neovascularization remains unclear, Herron et al. (1991) speculated that the new vessels play a sustaining or even a causal role in the pathophysiology of the aneurysm by secreting proteinases that destabilize the aortic matrix. Supportive evidence for this hypothesis comes from immunohistochemical studies in which proteinases such as gelatinase (matrix metalloproteinase 2, MMP-2) (Herron et al., 1991) and collagenase (MMP-1) (Irizarry et al., 1993) have been localized to the neovascular endothelium.

A highly relevant but poorly understood question is whether the neovascularization is an ongoing process in well-developed aneurysms. If neovascularization were ongoing it would signify the presence of active disease in the aneurysm wall and would support the hypothesis that the new vessels are critical in the

Ongoing angiogenesis in blood vessels of the abdominal aortic aneurysm
During angiogenesis, the αvβ3 integrin in adventitial vessels of aneurysms. The integrin αvβ3 has been proposed to play a critical role in angiogenesis. Brooks et al. have reported that this adhesion molecule is a marker for active angiogenic vessels in man and the chick, and that blocking its activity with a monoclonal antibody suppresses angiogenesis (Brooks et al., 1994).

During angiogenesis, the αvβ3 integrin uses the RGD sequence in the tenth fibronectin (III)-like domain of tenasin to bind to extracellular matrix (ECM) (Joshi et al., 1993; Sriramarao et al., 1993), and tenasin in turn has additional binding sites for matrix proteoglycans and heparin. Expression of αvβ3 is ubiquitous in the embryology and development of the tissue. In adult tissues, although the αvβ3 integrin may be detectable in low amounts (Suzuki et al., 1993), expression of tenasin isoforms is markedly down-modulated within one month of birth (Saga et al., 1991). Under abnormal circumstances, such as in tumorigenesis and wound healing, αvβ3 expression increases significantly (Suzuki et al., 1987; Felding-Habermann and Cheresh, 1993; Juhasz et al., 1993). Thus, detection of these proteins associated with the neovascularization, as we report in the present work, indicates the presence of active angiogenesis in aneurysms.

Materials and Methods

Reagents and antibodies
Rabbit anti-human αvβ3 polyclonal antibody R838, was purchased from Chemicon (Temecula, CA). Anti-endothelial nitric oxide synthase (eNOS) monoclonal antibody was purchased from Transduction Laboratories (Lexington, KY). Anti-human tenasin polyclonal antibody EHSB01 and monoclonal antibody TN2 were purchased from GibcoBRL Life Technologies (Gaithersburg, MD). For immunohistochemistry, secondary antibodies and substrates used were: goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) and diaminobenzidine (DAB) (DA KO Ltd, Copenhagen, Denmark) against R838 and eNOS, and goat anti-rabbit or mouse IgG antibodies conjugated with alkaline phosphatase and NTB/BCIP (Bioread, Hercules, CA) against EHSB01 or TN2. Other materials used were pepsin (Sigma, St. Louis, MO), Rnase-free DNase I and RNase (Gibco Life Tech., Gaithersburg, MD), RNase inhibitor (RNasin), and EZrTth RNA PCR kit (Perkin Elmer Corp., Norwalk, CT), digoxigenin-11-UTP and anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Manheim Corp., Indianapolis, IN), bio-11-dUTP and streptavidin-biotin alkaline phosphatase complex from ENZO (Farmindale, NY), ultrapure PCR water (Res. Gen., Huntsville, AL). αv and β3 primers purchased from Gibco Life Tech. Corp. (Gaithersburg, MD), were designed according to the published human av sequences (22,25). The av 5' primer, 5'-GACTGTTGGAAACATGTCG TAAACC, starts at position 1914 to 1943 of human av cDNA (HAV), and the αv 3’ primer, 5'-CCAGCTAAGAGTTGAGTT CCAGCC, from 2195 to 2219 of HAV. The β3 5' primer, 5'-TTTCGACGA GATCATGCA, starts at position 762 to 778 of human β3 cDNA (HIB3), and the β3 3' primer, 5'-AAAGTTCCGTTCGCTGTTGCA, from 1462 to 1485 of HIB3.

Immunohistochemistry
Aortas were obtained from 17 consecutive patients undergoing elective resection of AAA. All patients presented with abdominal aortic aneurysms of 5 cm in diameter size or greater. None of the patients suffered from rupture of AAA at the time of surgery. The sections of the AAA wall were reviewed macroscopically. Normal control aortic tissues were from 6 organ donors and 1 aorto-occlusive patient (ages average 38 yr; 4 male; 3 female). All tissues were fixed in buffered formalin (pH 7.4) for 16-24 h. Histological sectioning and immunohistochemistry were processed as in previous work (Fu et al., 1994). Briefly, aortic paraffin embedded tissue sections (4 μm thick) were dewaxed in xylene and absolute ethanol, inhibited for endogenous peroxidase activity in 0.5% hydrogen peroxide in absolute methanol (20 min), and blocked with 0.2% BSA to prevent nonspecific binding. The anti-αvβ3 antibody R838 (1:100), and the anti-tenasin antibodies, EHSB01 (1:50) and TN2 (1:200), were applied to the sections overnight at position 762 to 778 of human β3 cDNA (HIB3), and the β3 3’ primer, 5'-AAAGTTCCGTTCGCTGTTGCA, from 1462 to 1485 of HIB3.
Semi-quantitative morphometric analysis of microvessels

Quantitation of microvessel staining by anti-eNOS was carried out by an observer blinded to the source of each specimen, using three criteria: (1) overall impression of staining at 100X magnification, graded on a 0 to 4 scale (0 = minimal, 1 = easily detectable, 2 = moderate, 3 = extensive), (2) the number of capillaries identified in the most densely positive 100 x field, and (3) the number of endothelial cells identified in the most densely positive 400 x field.

Reverse transcription (RT) in situ PCR

For in situ PCR, we modified Nuovo's method (Nouvo, 1994) and used the EZ rTth RNA PCR kit as follows: after dewaxing and air drying, all sections were digested in 2 mg/ml pepsin in 0.01 N HCl (45 min, room temperature). The pepsin was inactivated by 1 min washes in ultrapure water and absolute ethanol, then the sections were air dried as a positive control. Amplification of tissue genomic DNA was performed by carrying slides directly on PCR cycles without digestion by DNAse I. For detection, the sections were chilled at 4 °C for 25 min at 62 °C. To control for nonspecific binding and background staining, after digestion of DNAse I, the slides were further digested by RNase H (50 U/ml in the digestive buffer) for 2 hours at room temperature.

For RT and in situ PCR processes, 50 µl of the reagent mixture (1 x EZ buffer, 200 µM of dNTP, 15 µM of -11-dUTP, 2.5 mM of Mn(Ac)₂ solution, 0.45 µM each of αₖ or β₃', 5' and 3' primers, 2.5 U/50 µl of rTth DNA Polymerase, and 60 U RNasin) was added to each section and sealed by the In Situ PCR assembly tool (Perkin Elmer Corp., Newark, CT). After incubation for 25 min at 62°C for reverse transcription, the PCR cycles were instituted in an in situ PCR Cycler (Ampligen PCR 1000, Perkin Elmer Corp., Newark, CT) under modified conditions (10 cycles: 94 °C for 10 s, 65 °C for 60 s and 72 °C for 120 s).

To determine the specificity of αₖ primers, we sampled aliquots of the supernatant on tissue sections immediately following the RT in situ PCR procedure, with or without treating the sections with DNase I. The aliquots were load on 1% agarose gel and Southern blotted by αₖ or β₃ probes. These probes (kindly provided by Dr. D. Shinar) were synthesized and labeled with biotin-11-dUTP by PCR amplification of rat αₖ (RAV 611) or β₃ (RIB 494/3) cDNA, using oligonucleotide primers (shown as above) (Shinar et al., 1993). The membrane was developed using Streptavidine-biotin-alkalinephosphatase and NBT/BCIP.

The sections were chilled at 4°C for 20 min, and blocked by 0.2% BSA in 0.1 SSC solution at 45°C for 10 min. Then the slides were incubated in goat anti-digoxigenin antibody conjugated with alkaline phosphatase for 30 min at room temperature. After 3 washes of Tris buffer (0.1 M, pH 7.5), the sections were treated for 10 min at room temperature with NBT (2.5 µl/ml)/BCIP (2.5 µl/ml) in Tris-buffered CaCl₂ solution (pH 9.5). The development was stopped by washing in running tap water. The sections were counterstained with Nuclear Fast Red, dehydrated and mounted for viewing.

Results

Microvessels detected by H&E and anti-eNOS

Histological sections of aortic aneurysms by H&E staining showed the presence of several hemorrhagic regions, large numbers of inflammatory cells and numerous microvessels (Figure 1). The inflammatory cells consisted mostly of macrophages and lymphocytes that were associated largely with the microvessels in the adventitia. In addition, some sub-intimal and medial regions showed the presence of neutrophils. By contrast, no inflammatory infiltrates or matrix abnormalities were evident in control aortas (Figure 1D).

Anti-eNOS immunohistochemical staining was employed for two reasons. First, detection of the intracellular eNOS enzyme in AAA and control tissue sections confirmed antigenic integrity of the preserved specimens. Second, anti-eNOS staining served as a marker for microvessel quantitation since the antibody detects the endothelial layer of both small and large microvessels.

In normal aortic wall, positive eNOS staining was seen in the endothelial cell layer of microvessels located in the adventitial vasa vasorum. The number of vessels seen per 100 x field was few, varying from none to 6. Intact medial smooth muscle cell nuclei were noted in most sections and medial elastin lamellae, as expected, were preserved in all control sections (Figure 2B). In AAA sections positive staining was again seen in the endothelial lining of microvessels. These vessels, however, were numerous compared to controls. Of the 10-30 vessels present per high power field (100 x), more than half were thin-walled capillaries of diameter < 10 µm and the remainders were thick-walled microvessels of diameter > 25 µm and wall thickness > 10 µm (Figures 2A and C). The capillaries were localized to both the subintimal region and the adventitia, while the thick-walled vessels occurred almost exclusively in the adventitia. Also noted in many sections were dense inflammatory infiltrates surrounding both thick-walled microvessels as well as capillaries. These cells,
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Based on their morphology on H&E staining, were mostly macrophages and lymphocytes. Lastly, elastic lamellae, in contrast to controls, were markedly disrupted and depleted. The results of the anti-eNOS morphometric analysis are shown in Figure 3. The differences between AAA and controls were striking. Mean capillaries were increased 15-fold per 100 × field (**P** < 0.001), and mean endothelial cells 8-fold per 400 × field (**P** < 0.001) in 17 AAA specimens by comparison to 7 controls. The general increase in staining for eNOS was highly remarkable in the AAA, although endothelial cells from control patients had approximately equivalent staining reactions on a per cell basis.

**Anti-αvβ3, anti-tenascin, and mRNA αv studies**

To determine the specificity of αv primers, we sampled aliquots of the supernatant on tissue sections immediately following the RT in situ PCR procedure, with or without treating the sections with DNase I. Then we blotted the aliquots using our αv probe. As shown in Figure 4, in non-aneurysm tissues a band was detectable only in the absence of DNase I treatment, which is expected because the probe can recognize a segment of non-digested genomic DNA (Figure 4, lane 1). In the presence of DNase I treatment, the band was detectable in aneurysm tissue (Figure 4, lane 2). No band was detectable in either non-aneurysm tissue (Figure 4, lane 3) or in aneurysm tissue that had been digested with both DNase I and RNase H (Figure 4, lane 4). These results confirmed that the αv probe specifically recognized the enhanced mRNA expression in aneurysm tissue.

The aortic wall distribution of the αvβ3 antigen is shown in Figure 5. In aneurysmal aortas, an overwhelming majority of microvessels, including capillaries, stained positively for the αvβ3 antigen. However, no staining occurred over the aortic endothelium, the medial and adventitial stroma, and in most large microvessels. A large number of macrophages, particularly those associated with microvessels, also stained positively. No staining was evident in either control aortas or in aneurysms in which R832 was replaced with non-immune rabbit serum as the primary antibody (negative control).

RT in situ PCR of tissue sections of aneurysms revealed co-localization of the αv mRNA with the αvβ3 antigen detected by immunohistochemistry (Figure 5).
mRNA staining was much more extensive than antigen staining. Almost all vessels, irrespective of diameter or location, most macrophages, and some lymphocytes, stained positively for \(\alpha_v\) mRNA. We studied \(\beta_3\) mRNA in one aneurysm specimen and found a distribution for \(\beta_3\) mRNA that closely resembled that of the \(\alpha_v\) mRNA (not shown). PCR products of \(\alpha_v\beta_3\) integrin were not evident in either non-vascularized regions of aneurysms or in non-aneurysmal control aortas (Figure 5). These results prove that both the transcription and the expression of the \(\alpha_v\beta_3\) integrin are upregulated in aneurysm microvessels. The distribution of tenascin staining around microvessels was closely linked to that of \(\alpha_v\beta_3\). Tenascin staining typically was seen circum-scribing thick-walled adventitial microvessels (Figure 6). Tenascin was also evident in subintimal regions and in areas of medial fibrosis.

**Discussion**

Although neovascularization of the aneurysm wall has been previously reported (Koch et al., 1990; Tilson...
Results of a Southern blotting of cDNA amplified with αv-specific primers (see Materials and Methods). Amplified fragments were then used for amplification by RT in situ PCR, using oligonucleotide αv-specific probes. DNA fragments were blotted onto a nitrocellulose membrane and hybridized with a biotin-11-dUTP labeled αv-specific probe, detected by streptavidin conjugated with alkaline phosphatase and NBT/BCIP. (see Materials and Methods). Lanes 1-4 same as in (A).

Figure 4. Presence of an amplified product of 308 bp encoding the genomic DNA of av integrin in human aortas and mRNA in AAA tissue. Dnase-untreated human aortas, AAA and normal aortic tissue were used for amplification by RT in situ PCR, using oligonucleotide primers (see Materials and Methods). Amplified fragments were then analyzed on a 1% agarose gel followed by Southern blotting. (A) Ethidium bromide staining of av-specific PCR-amplified fragments from aortic genomic DNA (lane 1), AAA tissue (lane 2), normal aortic tissue (lane 3) and Dnase- and Rnase-treated tissue (Lane 4). Samples used to generate data in lane 2 were treated with Dnase I in order to exclude the possibility of contamination of samples with genomic DNA. (B) Results of a Southern blotting of cDNA amplified with αv-specific oligonucleotide primers. DNA fragments were blotted onto a nitrocellulose membrane and hybridized with a biotin-11-dUTP labeled αv-specific probe, detected by streptavidin conjugated with alkaline phosphatase and NBT/BCIP. (see Materials and Methods). Lanes 1-4 same as in (A).

Figure 3. Results of morphometric semi-quantitative analysis of eNOS immunohistochemical studies. Evaluation was carried out using three criteria (see Methods). Mean values are expressed with standard errors. All bars shown were statistically significant at P < 0.001. Such findings underscore the striking increases in eNOS-staining microvessels observed in the AAA.

Angiogenesis in the aneurysm. Most extensive were thick-walled microvessels that displayed outer-medial to adventitial distribution, and thin-walled capillaries that invested the sub-intima. It is possible therefore, that vessels proliferating in the outer wall also developed transwall capillarization as part of a global neovascularization of the aneurysm. Two angiogenesis markers, namely the αβ3 integrin and tenascin, were widely detectable in these blood vessels. We conclude from these findings, that vascularization of the aneurysm is extensive and that new blood vessels form in the aneurysm wall as a result of ongoing angiogenesis.

Evidence for angiogenesis was particularly conspicuous in the immunohistochemistry and the RT in situ PCR studies. The αβ3 integrin, a vascular integrin of the cytoadhesin family (Albelda and Buck, 1990), is located in both the luminal and abluminal surfaces of endothelial cells as well in vascular smooth muscle cells, and has wide-ranging functions including cell adhesion and cell spreading (Albelda and Buck, 1990; Damjanovich et al., 1992; Joshi et al., 1993) and vascular permeability regulation (Tsuchida et al., 1995). We confirmed that in thick-walled microvessels, both the endothelial lining as well as the smooth muscle layers stained positively for the integrin. Recently, integrin αβ3 has been shown to be essential for angiogenesis and a mAb that blocks binding of ligands to αβ3, promoted tumor regression of angiogenic blood vessels in chick embryos (Brooks et al., 1994). The authors suggest that inhibition of ligand binding to αβ3 mediates removal of apoptotic cells by macrophages (Brooks et al., 1994; Flora and Gregory, 1994). Since apoptosis co-exists with growing tissue (Brooks et al., 1994), the presence of αβ3 expressing macrophages in the vicinity of αβ3 expressing vessels, further signifies that these locations were focal areas of active tissue growth and cell death.

From the experiments, two important results were, first, αmRNA was undetectable in control aortas but strongly detectable in vessels of the aneurysm wall; second, some aneurysm microvessels failed to stain for α although they stained positively for α mRNA. These findings indicate that increased transcription of the α gene is a significant feature of aneurysm microvessels. Increased transcription of the α and β genes are co-associated (Shinar et al., 1993), hence, transcription of β mRNA and β expression also increased in these aneurysms. This is indirectly indicated in these experiments, in the increased staining with a polyclonal antibody that recognizes the αβ3
dimer but not the individual subunits of the integrin (Suzuki et al., 1986). In one case, we determined the distribution of β3 mRNA by in situ PCR and found this distribution to be similar to that of αv mRNA (data not shown). The mismatch between negative avβ3 integrin staining and positive av mRNA expression in some areas indicates that increased αβ3 expression did not always follow increased gene transcription. This may reconcile the apparent inconsistency between our findings and those recently reported by Cheuk and Cheng (2004) in which no significant difference in RNA transcripts for αv and β3 were observed in AAA vs. control homogenates. Hence, it is possible that increased αβ3 expression is restricted to specific regions of active angiogenesis.

The immunohistochemical evidence for blood vessel-associated tenascin expression also supports our proposal that angiogenesis was active in these aneurysms. Tenascin is a large oligomeric glycoprotein that appears transiently in the extracellular matrix during tissue modelling and tissue repair (Tremble et al., 1994). Two domains of tenascin, both of which contain the integrin-recognizing arg-gly-asp (RGD) tripeptide sequence, support endothelial cell adhesion.
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(Joshi et al., 1993). One of these, the third fibronectin-III domain, also supports cell spreading (Sriramarao et al., 1993). The involvement of endothelial integrins and in particular, the \( \alpha_v\beta_3 \) integrin is indicated in that endothelial adhesion to tenascin is RGD dependent (Joshi et al., 1993) and is partly inhibited by anti-\( \alpha_v\beta_3 \) antibodies (Sriramarao et al., 1993). The ability of endothelial cells to adhere to tenasin (Joshi et al., 1993) suggests that endothelial tenascin receptors play a supportive role for establishing blood vessel structure during angiogenesis. The circumscribed perivascular tenasin expression seen here, is consistent with this notion and suggests that that tenasin provides a cylindrical template for vessel tube formation. Addition of tenasin to a fibronectin-based substratum upregulates the synthesis of three important matrix metalloproteinases in fibroblasts: collagenase (MMP-1), stromelysin (MMP-3), and gelatinase-B (MMP-9) (Newman et al., 1994; Newman et al., 1994). We have reported that these three matrix metalloproteinases are present in significantly higher levels in aneurysms than in control aortic specimens (Newman et al., 1994). Tenasin may promote aneurysm progression by inducing these metalloproteinases in endothelial cells of new blood vessels as well as in mesenchymal cells.

Using anti-eNOS Ab directed against endothelial cells, we observed marked increases in neo-vessel number in the aneurysm wall. Although no conclusions can be made concerning the possible role of eNOS or nitric oxide in aneurysm formation from our study, Kuhlencordt et al. have suggested that alterations in eNOS expression play a role in the formation of AAA (2001). Furthermore, we have shown that the nitric oxide by-product, nitrite, is capable of invoking deleterious changes in connective tissue proteins such as collagen and elastin (Paik et al., 2001). Since levels of nitrogen oxide gases are high in tobacco smoke and cigarette smoking is a strong environmental risk factor for AAA disease (Lederle et al., 2003), future studies will be directed at clarifying the role of nitric oxide and its by-products in the formation of AAA.

Finally, our finding that angiogenesis is active in the mature aortic aneurysm, suggests that a potential intervention site exists for pharmacologically reducing the rate of aneurysm enlargement or preventing the disease in genetically susceptible individuals. The observation by Brooks et al. (1994) that the angiogenesis in tumorigenesis can be arrested by antibody to \( \alpha_v\beta_3 \) suggests that anti-angiogenic therapy might be considered among other novel interventions that are presently under investigation, based on recent developments in our understanding of aneurysm pathogenesis.

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