

## Ongoing angiogenesis in blood vessels of the abdominal aortic aneurysm

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Abbreviations: AAA, abdominal aortic aneurysm; ECM, extracellular matrix; eNOS, endothelial nitric oxide synthase; MMP, matrix metalloproteinase

### 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  $\alpha_v\beta_3$  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 ~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 remodelling 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

progression of the aneurysm. We address this question through detection of the  $\alpha_v\beta_3$  integrin in adventitial vessels of aneurysms. The integrin  $\alpha_v\beta_3$  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  $\alpha_v\beta_3$  integrin uses the RGD sequence in the tenth fibronectin (III)-like domain of tenascin to bind to extracellular matrix (ECM) (Joshi *et al.*, 1993; Sriramarao *et al.*, 1993), and tenascin in turn has additional binding sites for matrix proteoglycans and heparin. Expression of  $\alpha_v\beta_3$  is ubiquitous in the embryology and development of the vascular system, and expression of members of the tenascin superfamily is similarly widespread in fetal tissue. In adult tissues, although the  $\alpha_v\beta_3$  integrin may be detectable in low amounts (Suzuki *et al.*, 1993), expression of tenascin isoforms is markedly down-modulated within one month of birth (Saga *et al.*, 1991). Under abnormal circumstances, such as in tumorigenesis and wound healing,  $\alpha_v\beta_3$  expression increases significantly (Suzuki *et al.*, 1987; Felding-Habermann and Cheresch, 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  $\alpha_v\beta_3$  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 tenascin polyclonal antibody EH5B01 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 (Bio-rad, Hercules, CA) against EH5B01 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).  $\alpha_v$  and  $\beta_3$  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'-GACTGTGTGGAAGACAATGTCTG TAAACCC, starts at position 1914 to 1943 of human av cDNA (HAV), and the  $\alpha_v$  3' primer, 5'-CCAGCTAAGAGTTGAGTT CCAGCC, from 2195 to 2219 of HAV. The  $\beta_3$  5' primer, 5'-TTTCGACGA GATCATGCA, starts at position 762 to 778 of human  $\beta_3$  cDNA (HIB3), and the  $\beta_3$  3' primer, 5'-AAGGTCCCGTCCCGTTGTTGCA, 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  $\mu$ m 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 non-specific binding. The anti- $\alpha_v\beta_3$  antibody R838 (1:100) and the anti-tenascin antibodies, EH5B01 (1:50) and TN2 (1:200), were applied to the sections overnight at 4°C. The anti-eNOS antibodies (1:2000) were applied to slides following a microwaving step used to enhance antigenicity. Following PBS washes, the sections were exposed to the appropriate secondary antibody for 1 h at room temperature. After PBS washes the sections were incubated for 10 min at room temperature with either DAB solution in PBS containing 0.01% hydrogen peroxide, or NBT/BCIP solution, depending on whether the secondary antibody was respectively, HRP or alkaline phosphatase linked. The sections were finally washed in running tap water, counterstained with hematoxylin for DAB or Nuclear Fast Red for NBT/BCIP, dehydrated in a reversed alcohol-xylene series, and mounted for semi-quantitative morphometric analysis, microscopy and photography.

### 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 1 to 4 scale (1 = minimal, 2 = easily detectable, 3 = moderate, 4 = extensive), (2) the number of capillaries identified in the most densely positive 100 × field, and (3) the number of endothelial cells identified in the most densely positive 400 × field.

### Reverse transcription (RT) *in situ* PCR

For *in situ* PCR, we modified Nuovo's method (Nuovo, 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 digested by RNase-free DNase I (400 U/ml in digestive buffer) overnight at 37°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 × EZ buffer, 200 µM of dNTP, 15 µM of -11-dUTP, 2.5 mM of Mn(AOC)<sub>2</sub> solution, 0.45 µM/each of  $\alpha_v$  or  $\beta_3$  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 60 s, 65 C for 60 s and 72 C for 120 s).

To determine the specificity of  $\alpha_v$  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 loaded on to 1% agarose gel and Southern blotted by  $\alpha_v$  or  $\beta_3$  probes. These probes (kindly provided by Dr. D. Shinar) were synthesized and labeled with biotin-11-dUTP by PCR amplification of rat  $\alpha_v$  (RAV 611) or  $\beta_3$  (RIB 494/3) cDNA, using oligonucleotide primers (shown as above) (Shinar *et al.*, 1993). The membrane was developed using Streptavidine-biotin-alkaline phosphatase 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 CaCl<sub>2</sub> 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.

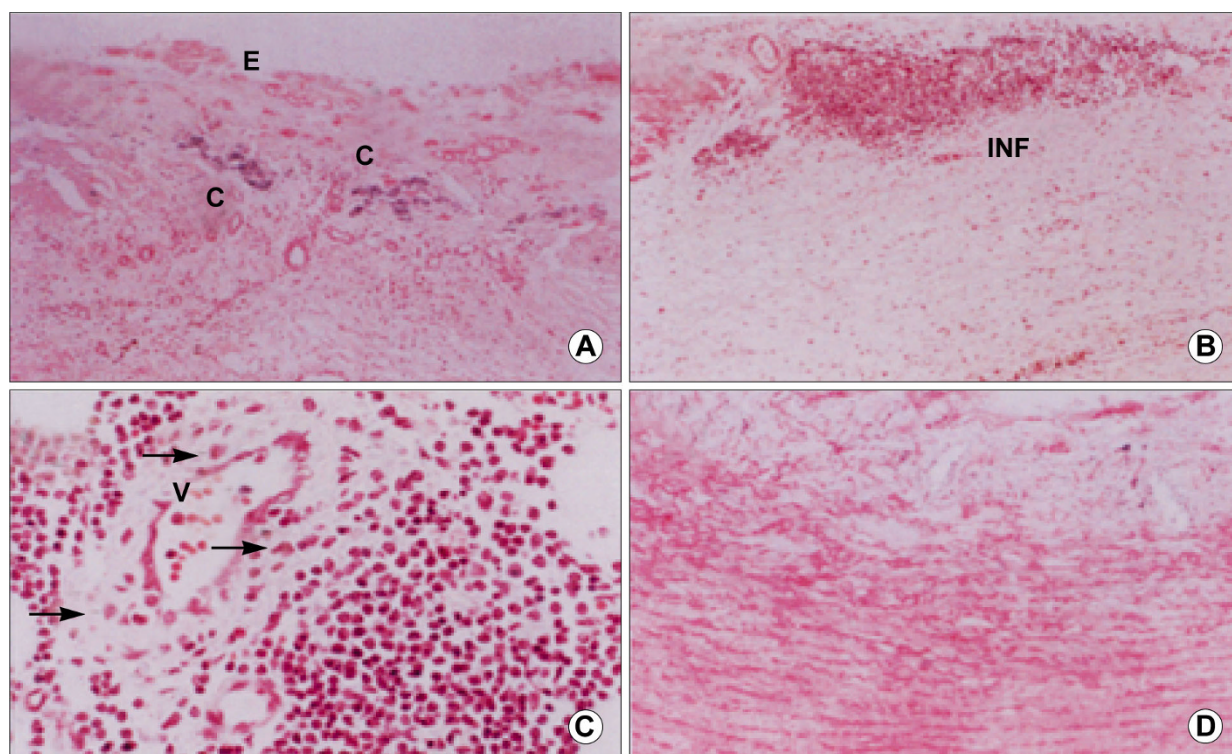
## Results

### 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 × 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 ×), 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,



**Figure 1.** Hematoxylin and Eosin staining. (A) low power view of aneurysm cross-section shows presence of multiple thin-walled capillaries (C) and intact endothelium (E) in the intima and sub-intima (40 $\times$ ). (B) The same section shows the presence of inflammatory infiltrate (INF), thick-walled vessel in the media and adventitia (40 $\times$ ). (C) high power view of aneurysm shows dense accumulation of macrophages (arrows) and lymphocytes associated with a thick-walled vessel (v) (100 $\times$ ); (D) low power view of normal non-aneurysmal aorta shows absence of infiltrates and modest amount of vascularization in the adventitia.

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  $\times$  field ( $P < 0.001$ ), and mean endothelial cells 8-fold per 400  $\times$  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.

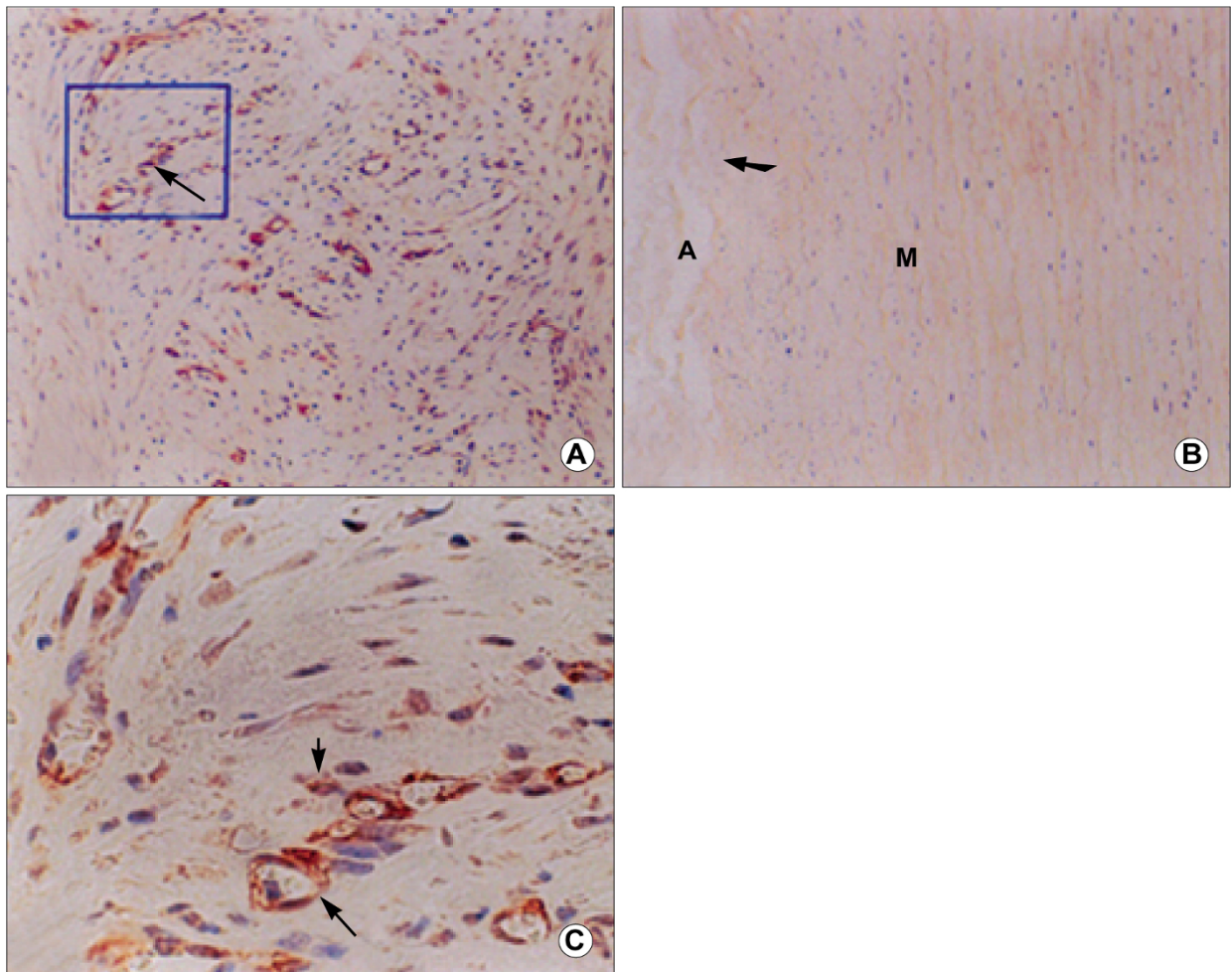
#### Anti- $\alpha_v\beta_3$ , anti-tenascin, and mRNA $\alpha_v$ studies

To determine the specificity of  $\alpha_v$  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  $\alpha_v$  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  $\alpha_v$  probe specifically recognized the enhanced mRNA expression in aneurysm tissue.

The aortic wall distribution of the  $\alpha_v\beta_3$  antigen is shown in Figure 5. In aneurysmal aortas, an overwhelming majority of microvessels, including capillaries, stained positively for the  $\alpha_v\beta_3$  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 R838 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  $\alpha_v$  mRNA with the  $\alpha_v\beta_3$  antigen detected by immunohistochemistry (Figure 5).



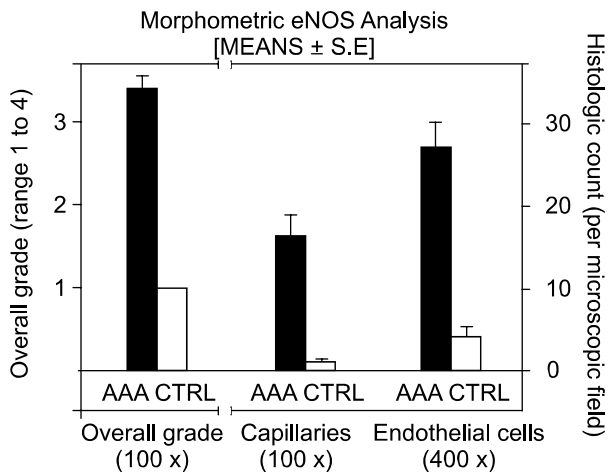
**Figure 2.** Anti-eNOS immunohistochemical staining (A) AAA wall at 40 × magnification. Most apparent are numerous eNOS-staining microvessels (arrow) outlined in brown stain. They are pictured infiltrating the AAA wall. Greater than 20 distinct lumens are identifiable. There is disarray of the normal regular elastic lamellar architecture (in comparison with B). Although the photomicrograph depicts the expected location for the adventitia/media interface, it is indiscernable. The arrow marks a single capillary, whose endothelial cell is better seen under higher magnification. (see panel C) (B) Non-aneurysmal aortic wall at 40 × magnification. Intact aortic media is free of microvessel invasion and hence dark brown eNOS staining is absent. The adventitia/media interface is clearly visible to the near left (arrow) and loose adventitial tissue (A) is seen to the far left. Long continuous strands of elastic tissue appear translucent, forming a regular orderly pattern within the media (M). Multiple smooth muscle cells with blue staining nuclei are seen within the aortic media interspersed between elastic lamellae. (C) 100 × magnification of boxed portion of panel A. An endothelial cell (large arrow) outlines a microvessel, staining dark brown with anti-eNOS antibody. Intraluminal translucent red and white blood cells are easily seen at this magnification. Cell nuclei are stained blue but are poorly visualized in endothelial cells that stain positive for eNOS. The tissue stroma contains a single mononuclear inflammatory cell (small arrow).

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

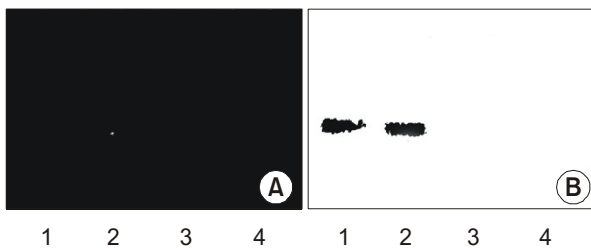
microvessels. The distribution of tenascin staining around microvessels was closely linked to that of  $\alpha_v\beta_3$ . Tenascin staining typically was seen circumscribing thick-walled adventitial microvessels (Figure 6). Tenascin was also evident in subintimal regions and in areas of medial fibrosis.

## Discussion

Although neovascularization of the aneurysm wall has been previously reported (Koch *et al.*, 1990; Tilson



**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.



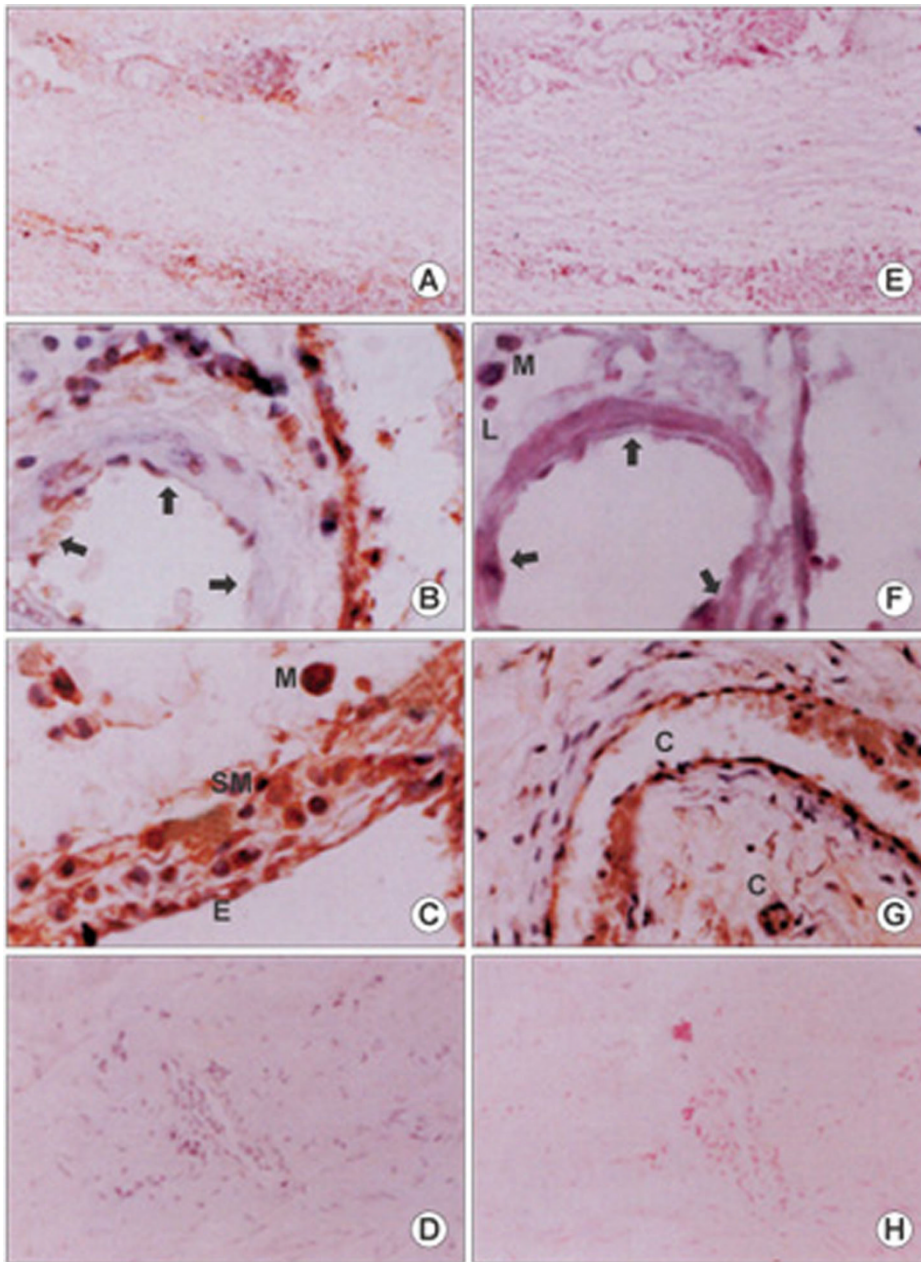
**Figure 4.** Presence of an amplified product of 308 bp encoding the genomic DNA of  $\alpha_v$  integrin in human aortas and mRNA in AAA tissue. Dnase-untreated human aortas, AAA and normal aortic tissue were used for amplification by RT In situ PCR, using oligonucleotide primers (see Materials and Methods). Amplified fragments were then analyzed on a 1% agarose gel followed by Southern blotting. (A) Ethidium bromide staining of  $\alpha_v$ -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  $\alpha_v$ -specific oligonucleotide primers. DNA fragments were blotted onto a nitrocellulose membrane and hybridized with a biotin-11-dUTP labeled  $\alpha_v$ -specific probe, detected by streptavidin conjugated with alkaline phosphatase and NBT/BCIP. (see Materials and Methods). Lanes 1-4 same as in (A).

*et al.*, 1990; Brophy *et al.*, 1991; Holmes *et al.*, 1995; Schneiderman *et al.*, 1995) the literature does not clarify whether new vessels appear early or late in the disease, or whether the vasculature undergoes continuous development and remodelling as the aneurysm enlarges. Our findings using an anti-eNOS Ab indicate that all layers of the aneurysm wall are vascularized and display characteristics typical of

angiogenesis. 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  $\alpha_v\beta_3$  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  $\alpha_v\beta_3$  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 (Tsukada *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  $\alpha_v\beta_3$  has been shown to be essential for angiogenesis and a mAb that blocks binding of ligands to  $\alpha_v\beta_3$ , promoted tumor regression of angiogenic blood vessels in chick embryos (Brooks *et al.*, 1994). The authors suggest that inhibition of ligand binding to  $\alpha_v\beta_3$  suppresses neovascularization and selectively promotes apoptosis of vascular cells. Interestingly,  $\alpha_v\beta_3$  expressing blood vessels in aneurysms of the present study, were typically associated with macrophages that also expressed both the  $\alpha_v\beta_3$  protein and  $\alpha_v$  mRNA. The  $\alpha_v\beta_3$  integrin 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  $\alpha_v\beta_3$  expressing macrophages in the vicinity of  $\alpha_v\beta_3$  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,  $\alpha_v$  mRNA was undetectable in control aortas but strongly detectable in vessels of the aneurysm wall; second, some aneurysm microvessels failed to stain for  $\alpha_v\beta_3$  although they stained positively for  $\alpha_v$  mRNA. These findings indicate that increased transcription of the  $\alpha_v$  gene is a significant feature of aneurysm microvessels. Increased transcription of the  $\alpha_v$  and  $\beta_3$  genes are co-associated (Shinar *et al.*, 1993), hence, transcription of  $\beta_3$  mRNA and  $\beta_3$  expression also increased in these aneurysms. This is indirectly indicated in these experiments, in the increased staining with a polyclonal antibody that recognizes the  $\alpha_v\beta_3$

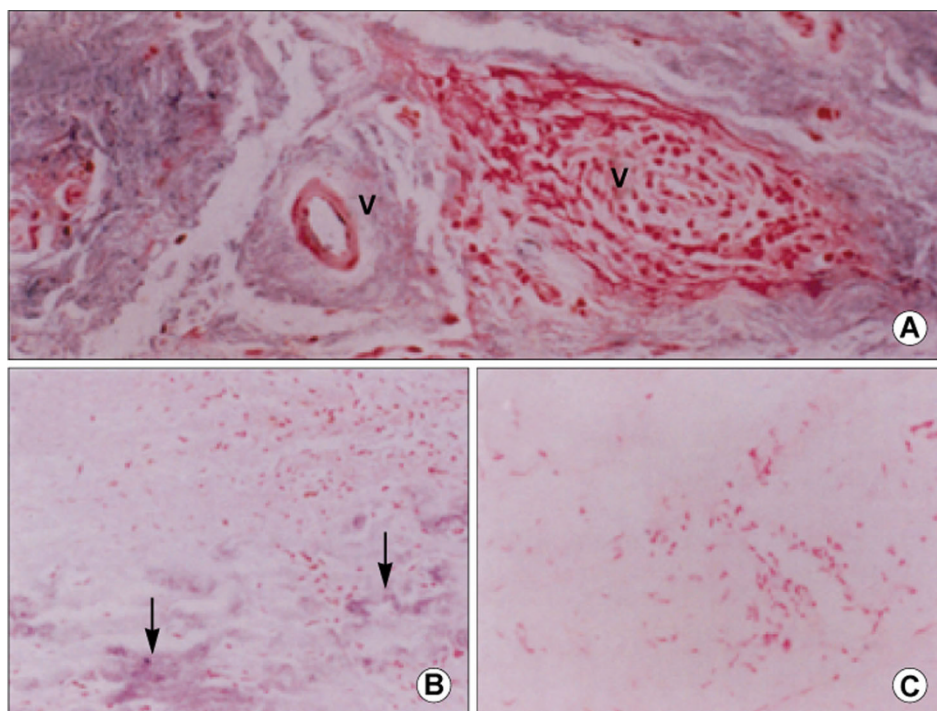


**Figure 5.** Demonstration of the  $\alpha_v\beta_3$  antigen and the  $\alpha_v$ mRNA in an aneurysm. A, B, C, D, G show the distribution of the  $\alpha_v\beta_3$  antigen determined by immunohistochemistry. E, F, H show  $\alpha_v$  mRNA distribution determined by reverse transcription (RT) and *in situ* PCR. (A, E) Identical 40 $\times$  views of an aneurysm cross-section. Panel A shows patchy deposition (brown stain) of the anti- $\alpha_v\beta_3$  immune product over vessels, smooth muscle and inflammatory cells. Panel E shows extensive blue/purple staining indicative of  $\alpha_v$  mRNA expression. (B, F) Identical 400 $\times$  views of a thick-walled microvessel. Arrows point to endothelium. This vessel failed to stain for  $\alpha_v\beta_3$  (panel B), although it showed endothelial expression of  $\alpha_v$  mRNA (panel F). Expression of  $\alpha_v$  mRNA in lymphocytes (L) and macrophages (M) is also shown in panel F. (C, G) 400 $\times$  views of a thick-walled vessel (panel C) and a capillary (c) [panel G] staining for  $\alpha_v\beta_3$ . Brown  $\alpha_v\beta_3$  staining is shown in the endothelial (E) and smooth muscle vessel layers (SM) as well as in perivascular macrophages (M) [panel C]. (D, H) 100 $\times$  view of control aorta shows absence of  $\alpha_v\beta_3$  staining (panel D) and lack of  $\alpha_v$  mRNA expression (panel H).

dimer but not the individual subunits of the integrin (Suzuki *et al.*, 1986). In one case, we determined the distribution of  $\beta_3$  mRNA by Rt *in situ* PCR and found this distribution to be similar to that of  $\alpha_v$  mRNA (data not shown). The mismatch between negative  $\alpha_v\beta_3$  integrin staining and positive  $\alpha_v$  mRNA expression in some areas indicates that increased  $\alpha_v\beta_3$  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  $\alpha_v$  and  $\beta_3$  were ob-

served in AAA vs. control homogenates. Hence, it is possible that increased  $\alpha_v\beta_3$  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 (Tremle *et al.*, 1994). Two domains of tenascin, both of which contain the integrin-recognizing arg-gly-asp (RGD) tripeptide sequence, support endothelial cell adhesion



**Figure 6.** Anti-tenascin immunohistochemical staining. (A) Section of an aneurysm shows distribution of blue/purple anti-tenascin immune product around blood vessel (V). Fast Red counterstaining shows cell distribution in the vessel wall, as well as inflammatory cells in an adjoining vessel. (B) Blue/purple discoloration shows tenascin distribution in the sub-intima (arrows). (C) Control aorta shows no staining for tenascin.

(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 tenascin (Joshi *et al.*, 1993) suggests that endothelial tenascin receptors play a supportive role for establishing blood vessel structure during angiogenesis. The circumscribed perivascular tenascin expression seen here, is consistent with this notion and suggests that tenascin provides a cylindrical template for vessel tube formation. Addition of tenascin 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). Tenascin 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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## References

Albelda SM, Buck CA. Integrin and other cell adhesion molecules. *FASEB J* 1990;4:2868

Brooks PC, Clark RAF, Cheresh DA. Requirement of vascular integrin  $\alpha_v\beta_3$  for angiogenesis. *Science* 1994a;264:569-71

Brooks PC, Montgomery AM, Rosenfeld MR, Reisfeld A, Hu T, Klier G, Cheresh DA. Integrin  $\alpha_v\beta_3$  antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* 1994b;79:1157-64

Brophy CM, Smith GJW, Tilson MD. Pathology of Non-specific Abdominal Aortic Aneurysm Disease. In "Current therapy in vascular surgery" (second edition) (Ernst CB, Stanley JC), pp. 238-41. B.C. Decker Inc, Philadelphia. 1991a

Brophy CM, Reilly JM, Smith GJW, Tilson MD. The role of inflammation in nonspecific abdominal aortic aneurysm disease. *Ann Vasc Surg* 1991b;5:229-33

Cheuk BLY, Cheng SWK. Differential expression of integrin  $\alpha_5\beta_1$  in human abdominal aortic aneurysm and healthy aortic tissues and its significance in pathogenesis. *J Surg Res* 2004;118:176-82

Damjanovich L, Albelda SM, Mette SA, Buck CA. Distribution of integrin cell adhesion receptors in normal and malignant lung tissue. *Am J Respir Cell Mol Biol* 1992;6: 197-206

Felding-Habermann B, Cheresh DA. Vitronectin and its receptors. *Curr Opin Cell Biol* 1993;5:864-8

Flora PK, Gregory CD. Recognition of apoptotic cells by human macrophages: inhibition by a monocyte/macrophage-specific monoclonal antibody. *Eur J Immunol* 1994;24:2625-32

Fu CZ, Jasani B, Vujanic GM, Leadbeatter S, Berry PJ, Knight BH. The immunocytochemical demonstration of a relative lack of nerve fibres in the atrioventricular node and bundle of His in the sudden infant death syndrome (SIDS). *Forensic Sci Int* 1994;66:175-85

Herron GS, Unemori E, Wong M. Connective tissue proteinases and inhibitors in abdominal aortic aneurysms. *Arteriosclerosis and Thrombosis* 1991;11:1667-77

Holmes D. R, Liao S, Parks WC, Thompson RW. Medial neovascularization in aneurysm: A histopathologic marker of aneurysmal degeneration with pathophysiological implications. *J Vasc Surg* 1995;21:761-72

Hynes RO. Integrin: A family of cell surface receptor. *Cell* 1987;48:549-54

Irizarry E, Newman KM, Gandhi RH, Nackman GB, Halpern V, Wishner S, Scholes JV, Tilson MD. Demonstration of interstitial collagenase in abdominal aortic aneurysm disease. *J Surg Res* 1993;54:571-4

Joshi P, Chung CY, Aukhil I, Erickson HP. Endothelial cells adhere to the RGD domain and the fibrinogen-like terminal

knob of tenascin. *J Cell Sci* 1993;196:389-400

Juhasz I, Murphy GF, Yan HC, Herlyn M, Albelda SM. Regulation of extracellular matrix proteins and integrin cell substratum adhesion receptors on epithelium during cutaneous human wound healing *in vivo*. *Am J Pathol* 1993; 143:1458-69

Koch AE, Haines GK, Rizzo RJ, Radosevich JA, Pope RM, Robinson PG, Pearce WH. Human Abdominal Aortic Aneurysms: Immunophenotypic analysis suggesting an immune-mediated response. *Am J Pathol* 1990;137:1199-219

Kuhlencordt PJ, Gyurko R, Han F, Scherrer-Crosbie M, Aretz TH, Hajar R, Picard M, Huang PL. Accelerated atherosclerosis, aortic aneurysm formation, ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice. *Circulation* 2001;104:448-54

Lederle FA, Nelson DB, Joseph AM. Smokers' relative risk for aortic aneurysm compared with other smoking-related diseases: a systematic review. *J Vasc Surg* 2003;38:329-34

Newman K.M, Ogata Y, Malon A. M. Irizarry, Gandhi E. R. H, Nagase H. and M.D.Tilson. Identification of matrix metalloproteinases 3 (stromelysin-1) and 9 (gelatinase B) in abdominal aortic aneurysm. *Arteriosclerosis and Thrombosis* 1994a;14:1315-20

Newman KM, Malon AM, Shin RD, Scholes JV, Ramey WG, Tilson MD. Matrix metalloproteinases in abdominal aortic aneurysm: Characterization, Purification, their possible sources. *Connective Tissue Res* 1994b;30:265-76

Nuovo GJ. PCR *in situ* hybridization protocols and applications (second edition). pp:1-416. Raven Press, New York. 1994

Paik DC, Dillon J, Galicia E, Tilson MD. The nitrite/collagen reaction: non-enzymatic nitration as a model system for age-related damage. *Con Tis Res* 2001;42:111-22

Saga Y, Tsukamoto T, Jing N, Kusakabe M, Sakakura T. Murine tenascin: cDNA cloning, structure and temporal expression of isoforms. *Gene* 1191;104:177-85

Schneiderman J, Bordin GM, Engelberg I, Adar R, Selfert D, Thinnis T, Bernstein EF, Dilley RB, Loskutoff DJ. Expression of Fibrinolytic Genes in atherosclerotic abdominal aortic aneurysm wall: a possible Mechanism for aneurysm expansion. *J Clin Invest* 1995;96:639-45

Shinar DM, Schmidt A, Halperin D, Rodan GA, Weinred M. Expression of  $\alpha_v$  and  $\beta_3$  integrin subunits in rat osteoclasts *in situ*. *J Bone Miner Res* 1993;8:403-14

Sriramarao P, Mendler M, Bourbon MA. Endothelial cell attachment and spreading on human tenascin is mediated by  $\alpha_2\beta_1$  and  $\alpha_v\beta_3$  integrins. *J Cell Sci* 1993;195:1001-12

Suzuki S, Argraves WS, Pytella R, Arai H, Krusius T, Pierschbacher MD, Rouslahti E. cDNA and amino acid sequence of the cell adhesion protein receptor recognizing vitronectin reveal a transmembrane domain and homologies with other adhesion protein receptor. *Proc Natl Acad Sci USA* 1986;83:8614-8

Suzuki S, Argraves WS, Arai H, Languino LR, Pierschbacher MD, Rouslati E. Amino acid sequence of the vitronectin receptor a subunit and comparative expression of adhesion receptor mRNAs. *J Biol Chem* 1987;262:14080-5

Suzuki S, Takahashi T, Nakamura S, Koike K, Ariyoshi Y, Takahashi T, Ueda R. Alteration of integrin expression in human lung cancer. *Jpn J Cancer Res* 1993;84:168-74

Tilson MD, Elefteriades J, Brophy CM. Tensile strength and collagen in abdominal aortic aneurysm disease. In "The cause and management of aneurysms" (R. M. Greenhalgh, Mannick JA, Powell JT), pp.97-104. W.B. Saunders Company, London. 1990

Tremle P, Chiquet-Ehrismann R, Werb Z. The extracellular matrix ligands fibronectin and tenascin collaborate in regulating collagenase gene expression in fibroblasts. *Mol Biol Cell* 1994;5:439-53

Tsukada H, Ying X, Fu CZ, Ishikawa S, McKeownALongo P, Albelda S, Bhattacharya S, Bray BA, Bhattacharya J. Ligation of the endothelial avb3 integrin increases capillary hydraulic conductivity of rat lung. *Circ Res* 1995;77:651-9