Vitronectin Expression and Interaction With Receptors in Smooth Muscle Cells From Human Atheromatous Plaque

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Abstract—Vitronectin (VN) is a plasma glycoprotein that promotes cell attachment and induces migration of human smooth muscle cells (SMCs) in culture. VN has been observed to accumulate in human atherosclerotic plaques, although its origin and role in atherosclerosis are not yet established. In the present experiments, synthesis of VN by intimal cells and its colocalization with receptors, \( \alpha v \beta 3 \) and \( \alpha v \beta 5 \), were studied by in situ hybridization and immunohistochemistry on 15 human atherosclerotic plaques from carotid arteries obtained after surgery. Strong VN protein and mRNA expression was observed in the intima and in the media. In the intima, VN mRNA expression was colocalized with SMCs, indicating that these cells produce VN, which may account for its accumulation in atherosclerotic plaques. In SMCs in culture, immunoprecipitation after metabolic labeling demonstrated that human SMCs do synthesize vitronectin. Confocal microscopic examination showed that VN colocalized with its receptors, \( \alpha v \beta 3 \) and \( \alpha v \beta 5 \), in the atherosclerotic intima. However, the distribution of the VN receptors on SMCs in culture in contact with VN was different. These observations suggest that VN plays various parts in atherogenesis via different SMC membrane receptors.

Key Words: vitronectin • vitronectin receptors • integrin • smooth muscle cells • atherosclerosis

Atherosclerosis is characterized by the development of an intimal thickening, which contain monocytes, T lymphocytes, and SMCs with an accumulation of lipid and extracellular matrix proteins.1 Medial SMCs during this process are activated, lose their differentiated phenotype, proliferate, and migrate into the intima. A number of reports have documented the major role of extracellular matrix proteins, particularly glycoproteins, in these pathological processes.2-4 VN is one of the glycoproteins accumulated in atherosclerotic plaques,5,6 although its origin and role in atherosclerosis have yet to be elucidated.

VN is present in plasma and serum at about 200 to 300 \( \mu g/mL \),7 but in common with other adhesive proteins, it is localized in the extracellular matrices of various tissues, including the vascular wall.8 The sites of VN synthesis in vivo are now being identified, with the liver emerging as a major site, although other normal9 and pathological tissues may synthesize VN.10 In the atherosclerotic plaques, the origin of VN could be due to the diffusion of plasmatic VN into the vascular wall, to VN release by activated platelets through damaged endothelium, or to the synthesis of cells participating in the atherosclerotic plaques.

VN is a multifunctional protein with a multiple binding domain that is thought to interact with a variety of plasma and cell proteins.8 VN has been shown to be involved in adhesion and migration of SMCs, neural crest cells, and keratinocytes.10-12 VN has also been shown to induce neurite outgrowth and differentiation13 and to promote myocyte differentiation in Drosophila embryos.14 In addition, VN may interact with several critical coagulation and fibrinolysis proteins. VN binds and stabilizes PAI-1 in its active conformation15,16 and binds to u-PA receptor,17 suggesting an additional role for VN in the control of protease activity. It has also been suggested that VN interacts with the complement activation system.8

VN contains an RGD sequence8 that intervenes in binding to integrins, cell surface receptors composed of noncovalently linked \( \alpha \) and \( \beta \) subunits.18 VN recognizes the integrins of the \( \alpha v \)-dependent system (\( \alpha v \beta 3 \), \( \alpha v \beta 5 \), \( \alpha v \beta 1 \), and \( \alpha v \beta 8 \) and the platelet receptor \( \alpha IIb \beta 3 \)).19 It has been demonstrated that VN induces migration of SMCs in vitro, which depends on \( \alpha v \beta 3 \) expression in these cells.15 \( \alpha v \beta 3 \) expression has recently been described in human arterial wall in vivo.20 Cellular contacts with extracellular matrix components via various integrins activate signal transduction pathways.21 These ultimately lead to alterations in gene expression, which underpin the changes in phenotype, state of differentiation, and migratory behavior of cells.22 Each of these events are thought to play a part in the development of atherosclerotic plaques1 and point to a role for VN and its receptors in this pathological process.

The present study was designed to find out which cell type produces VN in atherosclerotic plaques and to pinpoint the colocalizations between VN and its receptors, \( \alpha v \beta 3 \) and \( \alpha v \beta 5 \). We present evidence that SMCs express and synthesize VN in human carotid plaques. In addition, the distribution of VN was colocalized with its receptors, \( \alpha v \beta 3 \) and \( \alpha v \beta 5 \). In vitro studies...
were examined by confocal microscopy. Briefly, the slides were covered with streptavidin-horseradish peroxidase complex. After 30 minutes, the slides were rinsed in PBS, and coated slides (Sigma) were fixed in 2% PFA for 10 minutes at room temperature. Seven-micron cryosections were transferred to 3-aminopropyl triethoxysilane-coated slides. Sections were fixed in 4% PFA for 20 minutes, washed, and treated with proteinase K (1 μg/mL) in 0.1 M Tris-Cl (pH 7.5) and 0.05 mM EDTA for 20 minutes. The sections were rinsed in PBS, treated with glycine (2 mg/mL) for 2 minutes, then incubated in triethanolamine buffer, pH 8.5, for 5 minutes. After several washes, prehybridization buffer was applied for 30 minutes at 37°C. For hybridization, digoxigenin-labeled riboprobes were added, and hybridization was allowed to proceed overnight at 50°C. After hybridization, sections were incubated twice with 50% formamide and 2× SSC washes. The slides were immediately washed twice in 2× SSC, treated with RNase A (20 mg/mL) for 30 minutes, and washed twice in 2× SSC, followed by two 1× SSC washes. The slides were immediately processed for immunohistochemical staining with anti-digoxigenin alkaline phosphatase for 90 minutes. After several washes and saturation, the slides were incubated in 100 mM Tris-HCl, with 100 mM NaCl, 50 mM MgCl₂, and 1 mM levamisole for 5 minutes and rinsed in isopentane, prechilled in liquid nitrogen, embedded in OCT compound, and stored at −70°C. The cryosections were preincubated for 20 minutes in a blocking solution containing 5% BSA in PBS. Sections were first incubated with primary antibody at appropriate dilutions for 1 hour at room temperature, rinsed with PBS, incubated with biotinylated sheep anti-mouse Ig (Amersham) for 1 hour at room temperature, rinsed in PBS, and covered with streptavidin-horseradish peroxidase complex. After 30 minutes at room temperature, the sections were rinsed in PBS and revealed by incubation with 10% (vol/vol) 3,3′-diaminobenzidine tetrahydrochloride dihydrate in stable peroxide substrate buffer (In- terchim). A counterstain of 1% Harris hematoxylin was applied before cover-slipping. The following immunohistochemical controls were performed in each tissue: (1) no primary antibody and (2) preimmune mouse serum as primary monoclonal antibody. The following antibodies were used as cell markers on sections: anti-smooth muscle α-actin (1/1000, I4, Immunotech) to identify SMCs and HAM-56 (1/100, Becton Dickinson) to identify macrophages. An irrelevant isotype-matched immunoglobulin (Sigma) was used as negative control.

To define the colocalization of the VN with its receptors, samples were examined by confocal microscopy. Briefly, the slides were labeled with anti-integrin mAbs identified by anti-Ig mAb coupled with fluorescein isothiocyanate. After several washes and saturation, the slides were sequentially incubated with anti-VN polyclonal antibody, biotinylated sheep anti-mouse Ig, and finally with streptavidin-Texas Red complex (Amersham). The cover slips were mounted in mounting medium for fluorescence H-1000 (Vectashield, Vector Laboratories). Each image was taken at 0.4-μm intervals with a ×40 oil-immersion objective on a Diaphot TFD microscope. Each section was the average of five scans. The typical Z series is composed of optical sections in the x-y optical plane, while the Z series images were projected simultaneously to obtain the final image.

**Methods**

**Tissue Collection**

Fifteen specimens of plaque from the internal carotid arteries of patients who underwent surgery for transient ischemic attacks were obtained at surgery. Seven microns thick sections were cut on a cryostat for immunohistology. They were dried in air, stored at −20°C, and used within 24 hours. Immunohistochemistry and in situ hybridization. They were dried in air, stored at −20°C, and used within 24 hours. Immunohistochemistry was performed by the indirect immunoperoxidase method as previously described.23 Serial sections placed on 3-aminopropyl triethoxysilane-coated slides (Sigma) were fixed in 2% PFA for 10 minutes at room temperature. Endogenous peroxidase activity was blocked by incubating the sections in 0.3% hydrogen peroxide. Before staining, cryosections were preincubated for 20 minutes in a blocking solution containing 5% BSA in PBS. Sections were first incubated with primary antibody at appropriate dilutions for 1 hour at room temperature, rinsed with PBS, incubated with biotinylated sheep anti-mouse Ig (Amersham) for 1 hour at room temperature, rinsed in PBS, and covered with streptavidin-horseradish peroxidase complex. After 30 minutes at room temperature, the sections were rinsed in PBS and revealed by incubation with 10% (vol/vol) 3,3′-diaminobenzidine tetrahydrochloride dihydrate in stable peroxide substrate buffer (Interchim). A counterstain of 1% Harris hematoxylin was applied before cover-slipping. The following immunohistochemical controls were performed in each tissue: (1) no primary antibody and (2) preimmune mouse serum as primary monoclonal antibody. The following antibodies were used as cell markers on sections: anti-smooth muscle α-actin (1/1000, I4, Immunotech) to identify SMCs and HAM-56 (1/100, Becton Dickinson) to identify macrophages. An irrelevant isotype-matched immunoglobulin (Sigma) was used as negative control.

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**Cloning of VN cDNA and cRNA Preparation for In Situ Hybridization**

Total cellular RNA was prepared from confluent human SMC monolayer and was reverse-transcribed. VN-cDNA was amplified by PCR for 35 cycles at 62°C for annealing using primer sets according to the published cDNA sequence for VN20 (CAAGAAGTGGCAGTGTAAGTGA at position 16 and GAAGCTGAAGATGCCTCC at position 1019). The 854-bp PCR product obtained was purified, digested into a 650-bp fragment, and subcloned into pBluescript according to standard procedures. The specificity of the PCR product was verified by the DNA sequence of the insert determined using the dideoxy chain-termination DNA-sequencing method with modified T7 DNA polymerase. It corresponded to the published VN sequence.24,25 To construct the riboprobe, pBluescript containing the 650-bp-long human VN cDNA insert was linearized and in vitro-transcribed using T7 and T3 RNA polymerase for sense and antisense, respectively, in the presence of digoxigenin-labeled UTP.

**In Situ Hybridization**

Seven-micron cryosections were transferred to 3-aminopropyl triethoxysilane-coated slides. Sections were fixed in 4% PFA for 20 minutes, washed, and treated with proteinase K (1 μg/mL) in 0.1 M Tris-Cl and 0.05 mM EDTA for 20 minutes. The slides were then rinsed in PBS, treated with glycine (2 mg/mL) for 2 minutes, then incubated in triethanolamine buffer, pH 8.5, for 5 minutes. After several washes, prehybridization buffer was applied for 30 minutes at 37°C. For hybridization, digoxigenin-labeled riboprobes were added, and hybridization was allowed to proceed overnight at 50°C. After hybridization, sections were incubated twice with 50% formamide and 2× SSC at 55°C for 1 hour, washed twice in 2× SSC, treated with RNase A (20 mg/mL) for 30 minutes, and washed twice in 2× SSC, followed by two 1× SSC washes. The slides were immediately processed for immunohistochemical staining with anti-digoxigenin alkaline phosphatase for 90 minutes. After several washes and saturation, the slides were incubated in 100 mM Tris-HCl, with 100 mM NaCl, 50 mM MgCl₂, and 1 mM levamisole for 5 minutes and overnight at 37°C with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate solution in the same buffer. After washes, the slides were counterstained with Kernechetrol.

**Cell Culture**

SMCs were isolated from human adult aorta media by enzyme digestion as previously described31 and cultured in Ham’s F10 medium supplemented with 10% fetal calf serum, 5 mM HEPE, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37°C in a 5% CO₂/95% air atmosphere. The experiments were conducted on SMCs at the first passage.

**Immunofluorescence Microscopy**

Cellular adhesion assays were performed in eight-chamber slides coated with either human purified VN (10 μg/mL) or fibronectin (20 μg/mL) in PBS at 4°C overnight. The wells were then washed with PBS, and nonspecific adherence to the coat wells was blocked with 5% BSA in PBS for 2 hours at 37°C. Primary human SMCs were removed from the culture plates by brief incubation at 37°C with 0.5 mM EDTA, washed in PBS with Ca²⁺ and Mg²⁺, and resuspended in serum-free medium. The cells were placed in coated wells at a density of 50 000 cell/cm² in
serum-free medium. Synthesis of extracellular matrix proteins was inhibited by cycloheximide (25 μg/mL) 2 hours before and during adhesion assays. After 2 hours of adhesion on extracellular matrix proteins, the cells were fixed (2% PFA) and permeabilized with 0.2% Triton X-100. After saturation with 5% BSA in PBS, the cells were immunolabeled with anti-ανβ3, -αβ5, -β1, -αv subunit, or -vitronectin mAbs. The samples were then incubated for 1 hour with anti-lg mAb coupled with fluorescein isothiocyanate. For double immunofluorescence, after application and labeling of the first mAbs as above, the samples were then incubated for 1 hour with a focal contact marker, anti-vinculin (1/50) mAb, followed by biotinylated sheep anti-mouse Ig and finally with streptavidin–Texas Red complex for 30 minutes. The coverslips were mounted in mounting medium for fluorescence H-1000 and examined with a Microphot-FXA microscope.

Immunoprecipitation and Immunoblotting

For immunoprecipitation experiments, primary SMCs were preincubated for 24 hours in serum-free medium, for 1 hour in methionine-free medium, and metabolically labeled with (35S) methionine and (35S) cysteine (100 μCi/mL) for 18 hours in serum-free medium. The cell supernatants were collected and clarified by centrifugation. The cells were lysed in RIPA buffer (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 1% Triton X-100, 1% deoxycholate, 1 mM EDTA, and protease inhibitors) at 4°C. Both culture media and cell lysates were precleared for autodigestion and immunoprecipitated with polyclonal or monoclonal antibodies anti-VN in a rotary shaker overnight, followed by absorption with protein A-Sepharose for 3 hours at 4°C. As control, VN antibodies were preabsorbed on purified VN (10 μg/mL). The Sepharose particles were collected by centrifugation and washed three times in RIPA buffer and twice in PBS and water. The immunocomplexes were eluted from protein A-Sepharose by boiling in Laemmli buffer and analyzed by 7.5% SDS-polyacrylamide gel electrophoresis under reducing conditions. The gel was either dried and exposed for autoradiography or electrophoretically transferred onto nitrocellulose membranes for protein immunoblotting. In this case, the membranes were saturated with 5% BSA and incubated with anti-VN mAb for 1 hour. After several washes, the bound antibody was detected with biotinylated sheep anti-mouse Ig and finally with streptavidin-horseradish peroxidase complex. The immunoblots were developed with the chemiluminescence detection system according to the manufacturer’s recommendations (Renaissance, DuPont New England Nu-clear). Relative mass was determined using high molecular mass standards (Bioabs, New England).

Flow Cytometry

Expression of αν, β1 subunits, and αβ3 and αβ5 integrins on human primary SMCs was determined by flow cytometry. Confluent SMCs were harvested by brief incubation in buffer (6 mM glucose, 5.3 mM KCl, 125 mM NaCl, 18 mM HEPES, 0.1× PBS) with 0.5 mM EDTA. After washes in PBS (with Ca2+ and Mg2+) the cells were incubated with mAbs in PBS for 1 hour at 4°C, washed three times in cold PBS, and resuspended in fluorescein isothiocyanate-conjugated goat anti-mouse Ig for 1 hour at 4°C. After three additional washes, the samples were fixed in 1% PFA. Measurements were made in a Coulter type XL flow cytometer. The mean of the fluorescence channels was evaluated by the computer for each sample. The x axis represents the fluorescence intensity, and the y axis represents the cell number; 10 000 events were analyzed for each sample.

Results

VN Expression in Human Carotid Plaques

We analyzed VN expression on human atherosclerotic plaques obtained from specimens of internal carotid artery after endarterectomy. Fig 1 shows two human plaques at different stages of progression: a fibrotic plaque comprising SMCs and some macrophages (Fig 1, A through D) and a fibroatheroma lesion with macrophage infiltration and a lipid core (Fig 1, E through G). Serial sections of endarterectomy fragments were stained for both VN and cell-specific markers for SMCs, smooth muscle α-actin (Fig 1, A and E), macrophages (HAM-56) (Fig 1, C and G), and T lymphocytes (anti-CD3 UCHT1, Immunotech) (data not shown). VN labeling was distributed throughout the media of normal and atherosclerotic arteries (Fig 1, B, F, and H). A punctiform VN staining was observed in the intimal thickening of cellular fibrotic plaques (Fig 1, B and F), contrasting with large diffuse masses of VN in the necrotic core of fibroatheroma lesions (Fig 1, F). We found the same labeling with a second antibody, a polyclonal anti-VN. Moreover, preabsorption of mAb anti-VN with purified VN eliminated the pattern observed on carotid arteries (data not shown). It can be seen on Fig 1, B and F, that the intimal VN was mainly expressed on α-actin-positive areas, suggesting an association with intimal and medial SMCs. In the fibrous cap, VN labeling was colococalized with HAM-56 staining (Fig 1, F). These results indicated that VN expression was associated with SMCs and sometimes with macrophages in the intimal thick-
performing. Primary human SMCs were cultured until confluence on medium supplemented with serum. At confluence, SMCs were maintained for 24 hours in serum-free medium, for an additional hour in methionine-free medium, then labeled with 100 μCi/mL of 35S-methionine/35S-cysteine medium. The supernatants of cultured SMCs and the cell lysates were immunoprecipitated with a polyclonal anti-VN antibody. On autoradiography (Fig 3, A), two bands identified at 65 and 75 kd were detected. The same results were obtained when the immunoprecipitation was performed with mAb anti-VN. Moreover, the precipitation of 75- to 65-kd polypeptides was inhibited by preabsorption of the antibody with purified VN (data not shown). Immunoblotting with mAb anti-VN on the same membrane revealed two bands that migrated at the same positions as purified human VN (Fig 3, B). The majority of VN protein was immunoprecipitated from the cell lysate, while only a small amount was found in SMC supernatant. Low-molecular-weight polypeptides were detected, suggesting that VN was proteolytically cleaved from the larger form. On immunofluorescence with mAb anti-VN, a fibrillary pattern of VN deposits was observed on surface membranes of SMCs (Fig 3, C).

Integrin Expression in Human Carotid Plaques
Because VN interacts with different membrane integrin receptors, we looked for αvβ3 and αvβ5 on human carotid plaques. Both αvβ3 and αvβ5 integrins were expressed in intimal thickening and media (Fig 4, A and B, respectively). On serial sections, αvβ3 colocalized with α-actin staining in the intima (Fig 4, D). Expression of αvβ5 was less pronounced than that of αvβ3 in media but stronger in intima of human atherosclerotic plaques (Fig 4, B). Interestingly, αvβ5 staining colocalized with both α-actin and HAM-56 staining (Fig 4, D and E, respectively). In normal arteries, SMCs expressed both αvβ3 and αvβ5 (data not shown). On serial sections, VN expression (Fig 4, C) was observed in both the fibrotic acellular part of atherosclerotic plaques and in cellular areas. At higher magnification, VN was clearly colocalized with αvβ3 on the internal side of the cellular rim of the necrotic core (Fig 4, A), contrasting with the more external expression of αvβ5 on the rim (Fig 4, B). VN thus appeared to interact with both αvβ3 and αvβ5 in human atherosclerotic plaques.

To pinpoint the interactions between VN and its receptors in situ, carotid plaques were double immunostained for VN and integrins and examined by confocal microscopy. Colocalization of red-labeled VN with green-labeled receptors was indicated by a yellow coloration. It can be seen in Fig 5 that VN was colocalized with both αvβ3 (Fig 5, A) and αvβ5 (Fig 5, B) integrins on 0.4-μm-sections of carotid tissue specimens. In contrast, the colocalization between VN and the β1 subunit in the same area was sparse and scattered in comparison with the massive αvβ3/VN and αvβ5/VN colocalization (Fig 5, C).

Integrin Expression In Vitro
Integrin expression on human aortic SMCs in culture was analyzed by flow cytometry (Fig 6). It can be seen in Fig 6, B, that SMCs strongly expressed the αv subunit. Analysis of the β
The integrin subunit associated with \( \alpha_v \) showed a higher expression of the \( \alpha_v \beta_5 \) than the \( \alpha_v \beta_3 \) integrin. We did not obtain any direct evidence for \( \alpha_v \beta_1 \) complex expression, although a moderate expression of the \( \beta_1 \) subunit was observed, comparable to that of \( \alpha_v \beta_3 \), indicating a relationship between \( \alpha_v \beta_1 \) and \( \alpha_v \) expression.

To determine the distribution of SMC receptors on the membrane surface, cells were placed on VN-coated plates in the presence of cycloheximide to inhibit the synthesis of extracellular matrix proteins. After 2 hours, the cells were

integrated and stained with a focal contact marker, anti-vinculin mAb (Fig 7, E through G), along with mAb against the \( \alpha_v \) subunit (Fig 7, A), \( \alpha_v \beta_3 \) (Fig 7, B), or \( \alpha_v \beta_5 \) (Fig 7, C) complexes. In the presence of cycloheximide, SMCs were seen to organize focal contacts, as shown by vinculin staining (Fig 7, E through G). As described by Burridge et al., we observed an exclusion of the ligand-specific antibody, anti-VN mAb, from focal contacts on cells attached to the VN coat (Fig 7, D). This pointed to an involvement of VN in focal contact organization.
Furthermore, the two VN receptors were distributed differently on SMC surface membranes in contact with VN. $\alpha v\beta 3$ expression was low and punctiform (Fig 7, B) and not associated with focal contact, evidenced by mAb to vinculin (Fig 7, F). In contrast, $\alpha v\beta 5$ staining was both punctiform and in focal contact (Fig 7, C) and codistributed with vinculin staining (Fig 7, G). The $\alpha$ subunit pattern revealed a strong focal contact staining (Fig 7, A) as well as a punctate nonfocal staining on some cells. In control experiments with SMCs placed on fibronectin, $\alpha v\beta 3$ expression was in focal contact in association with vinculin staining, whereas $\alpha v\beta 5$ staining was diffuse (data not shown). No labeling was detected in the absence of primary antibody.

**Discussion**

VN is a multifunctional glycoprotein that appears to play roles in the migration, attachment, and differentiation of cells.\textsuperscript{11,13} The immunohistochemical and hybridization study presented here provides evidence for the expression of VN in human atherosclerotic arteries. VN protein deposits were observed in fibrous plaques and arterial media. Strong labeling was observed deep in the intimal thickening, near the internal elastic lamina. Moreover, VN seemed to be localized around and sometimes within the necrotic core. In the cellular areas of intimal thickening, most VN protein colocalized with the SMC marker $\alpha$-actin, suggesting that much of the VN was associated with SMCs in both atherosclerotic plaques and normal media. In addition, some VN protein was colocalized with macrophage HAM-56 staining. Although VN protein was present in normal media, its expression was markedly elevated in the intima of human atherosclerotic plaques. These results are in line with the accumulation of VN observed by numerous authors in human atherosclerotic plaques associated with cell debris, collagen, and elastin and with an activation of complement.\textsuperscript{6,27} In experimental models, an accumulation of VN expression in arterial wall with no increase in plasma VN has been described in both hypercholesterolemic and mechanically injured aortas\textsuperscript{28} as well as in Watanabe heritable hyperlipidemic rabbits.\textsuperscript{29}

An accumulation of VN protein within the plaque was demonstrated immunohistochemically, although the source of

![Figure 4](image1.png)

**Figure 4.** Integrin expression on carotid artery plaques. Serial sections of human carotid arteries were immunostained with mAb anti-$\alpha v\beta 3$ (A), $\alpha v\beta 5$ (B), VN (C), $\alpha$-actin (D), and HAM-56 (E) (original magnification: A through E, $\times 5$; F through H, $\times 20$). i indicates intima; m, media; nc, necrotic core; and arrows, internal elastic lamina.

![Figure 5](image2.png)

**Figure 5.** Confocal analysis. Colocalization of VN (red) and VN receptors (green), $\alpha v\beta 3$ (A), $\alpha v\beta 5$ (B), and $\beta 1$ (C) were studied after double immunofluorescence on intimal thickening of human carotid arteries. VN and its receptors were visualized on tissue sections by confocal microscopy. All fluorescence images are the average of five frames per 0.4 $\mu$m optical section. The colocalization of VN and its receptor was observed in yellow from superimposition of the red and green staining.

![Figure 6](image3.png)

**Figure 6.** VN receptor expression on cultured SMCs. Human SMCs were immunolabeled with either primary antibody (A) or antibody against the $\alpha v$ (B), $\alpha v\beta 3$ (C), $\alpha v\beta 5$ (D), $\beta 1$ (E), or $\alpha 5$ (F) subunits. A fluorescent secondary antibody was applied, and 10 000 cells were analyzed by flow cytometry. The x axes represent fluorescent intensity, and the y axes number of cells. Identical results were obtained in three independent experiments.
the arterial VN was not clear. It may have diffused from plasma, been secreted by activated platelets or been synthesized by cells in the plaque. To determine whether cells in atherosclerotic lesions synthesize VN and whether intimal SMCs remodel their extracellular matrix via VN, we carried out an immunolabeling and in situ hybridization study of human atheromatous carotid arteries. We found that a large population of SMCs and a few macrophages expressed VN mRNA. It is of interest that the VN mRNA expression was not restricted to pathological intimal SMCs, because some expression was also observed in medial SMCs. Therefore, normal medial SMCs as well as SMCs in the intima appear to synthesize VN, which would account for its presence in the arterial wall. Some of the VN in atherosclerotic plaques may be derived from increased VN synthesis by SMCs induced by cytokines or growth factors secreted by cells in the plaque. To determine whether cells in atherosclerotic plaques.41,42 We found that the VN receptors were also detected and were thought to be proteolytic fragments. In this respect, it has been reported that VN can be cleaved and degraded by various enzymes. For example, plasmin cleaves VN into 61- to 63-, 56-, 42-, and 35-kd polypeptides.33 The small amounts of VN protein detected in SMC supernatant suggested that most of the synthesized VN was associated with cells. The immunofluorescence labeling studies showed that it was anchored on the membrane surface in a fibrillary distribution. We therefore concluded that SMCs produce and deposit their own VN.

With respect to cell-matrix interactions, VN interacts with the cell surface via integrins containing the αv subunit.34,35 This integrin subunit is associated with multiple β chains, including β1, β3, β5, and β8. Among these heterodimers, αvβ3, αvβ5, and αvβ1 are expressed on SMCs.36,37 We examined the localization of VN-binding integrins, αvβ3 and αvβ5, on human carotid arteries, and we present evidence that both receptors are expressed in human atherosclerotic plaques. The intimal and medial expression of αvβ3 in human arteries has been reported by Hoshiga et al.20 We present here the first evidence of αvβ5 expression in atherosclerotic lesions. On serial sections in situ, αvβ5 integrin appeared to be expressed by both SMCs and macrophages, which is consistent with the demonstration of αvβ5 expression by macrophages38 and SMCs in vitro.4 Interestingly, αvβ3 and αvβ5 staining was observed in VN-positive areas. To obtain more evidence of a colocalization of VN with its receptors, sections of artery were examined by confocal microscopy. VN was observed together with αvβ3 and αvβ5 receptors in the fibrous cap of atherosclerotic lesions. These results were supported by the in vitro findings that human cultured SMCs express both αvβ3 and αvβ5 and adhere to VN. However, it can be seen in Figure that not all αvβ3 and αvβ5 integrins were associated with VN ligand. It is known that αvβ3 and αvβ5 interact with several ligands, including fibronectin and osteopontin.4,39,40 These extracellular matrix proteins are all expressed in atherosclerotic plaques.41,42 We found that the VN receptors αvβ3 and αvβ5 and the αv integrin subunit exhibited different patterns of expression in contact with VN on the surface of SMCs. The αvβ3 integrin was observed in a punctiform and diffuse
distribution on VN and was not concentrated in focal contacts. This is not in agreement with the findings of Clyman et al., but they used a mAb against β3 subunit and ductal SMCs rather than the arterial SMCs used in the present study. In contrast to the distribution of αvβ3, αvβ5 was observed in both punctiform and in focal adhesion sites of the SMC-VN interactions, contrasting with the diffuse distribution on fibronectin. So, SMC αvβ5 can be organized in focal adhesion plaque in contact with VN. Similarly, the αv and β1 subunits (data not shown) were distributed in focal contact, suggesting that αvβ1 was abundant in focal adhesion sites, although we were unable to precisely localize the complex αvβ1 itself. The participation of β1 subunit integrin in focal adhesion sites on SMCs has been reported by others. αvβ3 expression on SMCs has been demonstrated to be involved in SMC migration. The functional roles of the αvβ5 integrins on SMCs are as yet unknown. For instance, it has been demonstrated that keratinocytes migrate on VN via αvβ5 only after activation by growth factors. Many extracellular matrix proteins have been shown to transmit signals via integrins, giving rise to a variety of intracellular processes, including tyrosine phosphorylation, an increase in intracellular pH, and calcium flux. The distributions of VN receptors on the cell surface as well as in atherosclerotic plaques suggest that various functional changes may be induced by VN in SMCs via different signal transducers.

The pathophysiological significance of VN in atherosclerotic plaques is still unclear, although several lines of evidence point to a role for VN in the cellular processes involved in atherogenesis. Migration of SMCs into the intima layer is an important contributor to the intimal thickening in atherosclerotic lesions, and it has been shown that VN mediates chemotactic and haptotactic activities of SMC in vitro. Furthermore, the migration on VN is dependent on the vitronectin receptor, αvβ3, which is strongly expressed in human atherosclerotic plaques. VN could thus play a role in the recruitment of SMCs from the media to the intima. Peptides that bind to αvβ3 receptor have been found to reduce neointima formation after balloon injury in rabbit and hamster arteries. Although αvβ3 binds several extracellular matrix proteins, VN could be involved in the regulation of cellular recruitment. Interestingly, PAI-1, which is highly expressed in human atherosclerotic plaques, may block SMC migration on VN by hindering its access to the VN receptor. Indeed, PAI-1-binding sites for VN overlap the region containing RGD cell attachment sites for the VN receptor. The VN synthesized and secreted by SMCs may thus modulate its effect on migration as a function of its affinities for the two substrates, PAI-1 or the integrin αvβ3. Moreover, because VN stabilizes PAI-1 in extracellular matrix proteins, thereby protecting them from plasminogen activator-mediated degradation, it may help stabilize plaques.

In conclusion, we show here that (1) VN protein and mRNA are expressed in human carotid artery plaques; (2) SMCs synthesize VN in the plaque, which may account for its accumulation; (3) αvβ3 and αvβ5 integrins are expressed in these plaques and colocalize with VN in the intima; and (4) these integrins were differently distributed on the membrane surface. Comprehension of the true functions of VN and its interactions with its receptors in atherosclerotic lesions will have to await the results of further studies.

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Vitronectin Expression by Smooth Muscle Cells


Vitronectin Expression and Interaction With Receptors in Smooth Muscle Cells From Human Atheromatous Plaque
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