α5β1 Integrin Signaling Mediates Oxidized Low-Density Lipoprotein–Induced Inflammation and Early Atherosclerosis

Arif Yurdagul Jr, Jonette Green, Patrick Albert, Marshall C. McInnis, Andrew P. Mazar, A. Wayne Orr

Objective—Endothelial cell activation drives early atherosclerotic plaque formation. Both fibronectin deposition and accumulation of oxidized low-density lipoprotein (oxLDL) occur early during atherogenesis, and both are implicated in enhanced endothelial cell activation. However, interplay between these responses has not been established. The objective of our study was to determine whether endothelial matrix composition modulates the inflammatory properties of oxLDL.

Approach and Results—We now show that oxLDL-induced nuclear factor-κB activation, proinflammatory gene expression, and monocyte binding are significantly enhanced when endothelial cells are attached to fibronectin compared with basement membrane proteins. This enhanced response does not result from altered oxLDL receptor expression, oxLDL uptake, or reactive oxygen species production, but results from oxLDL-induced activation of the fibronectin-binding integrin α5β1. Preventing α5β1 signaling (blocking antibodies, knockout cells) inhibits oxLDL-induced nuclear factor-κB activation and vascular cell adhesion molecule-1 expression. Furthermore, oxLDL drives α5β1-dependent integrin signaling through the focal adhesion kinase pathway, and focal adhesion kinase inhibition (PF-573228, small interfering RNA) blunts oxLDL-induced nuclear factor-κB activation, vascular cell adhesion molecule-1 expression, and monocyte adhesion. Last, treatment with the α5β1 signaling inhibitor, ATN-161, significantly blunts atherosclerotic plaque development in apolipoprotein E–deficient mice, characterized by reduced vascular cell adhesion molecule-1 expression and macrophage accumulation without affecting fibrous cap size.

Conclusions—Our data suggest that α5β1-mediated cross-talk between fibronectin and oxLDL regulates inflammation in early atherogenesis and that therapeutics that inhibit α5 integrins may reduce inflammation without adversely affecting plaque structure. (Arterioscler Thromb Vasc Biol. 2014;34:1362-1373.)

Key Words: atherosclerosis ■ fibronectins ■ inflammation ■ integrin alpha5beta1 ■ lipoproteins, LDL

Hypercholesterolemia, an increase in circulating cholesterol, remains the most recognized atherogenic risk factor, with genetic and epidemiological studies linking low-density lipoproteins (LDLs) to atherosclerosis. While native LDL acts benignly on vascular pathology, post-translational modifications such as oxidation and glycation enhance the atherogenic nature of LDL. Modified LDL exhibits reduced LDL receptor affinity and enhanced uptake by scavenger receptors (i.e., CD36 and the lectin-like oxidized LDL [oxLDL] receptor 1). Although the role of oxLDL in atherosclerosis has yet to be definitively proven, multiple lines of evidence suggest that oxLDL contributes to endothelial cell activation, a phenotypic conversion characterized by enhanced permeability and proinflammatory gene expression. Although early reports failed to show oxLDL-induced proinflammatory gene expression in endothelial cells, subsequent studies demonstrated that oxLDL stimulates vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) expression to facilitate leukocyte adhesion. Apolipoprotein E-deficient (ApoE−/−) mice lacking the endothelial oxLDL receptor 1 exhibit reduced plaque size, whereas enhancing endothelial oxLDL receptor 1 expression exacerbates atherosclerotic plaque development.

Current data from both cell culture and animal models suggest that extracellular matrix remodeling regulates endothelial cell activation. Although the macrovasculature is largely devoid of fibronectin under healthy conditions, the subendothelial basement membrane remodels into a fibronectin-rich matrix simultaneously with enhanced endothelial cell proinflammatory gene expression during early atherogenesis. Endothelial cells interacting with fibronectin show augmented inflammatory responses to shear stress, and limiting fibronectin deposition in atheroprone mice either genetically or with peptide inhibitors blunts both inflammation and early atherosclerotic plaque formation.

OxLDL promotes matrix remodeling during multiple pathological conditions; however, much less is known about how matrix composition affects the cellular response to oxLDL.
Early studies found that minimally modified LDL could activate endothelial β1 integrins to promote apical fibronectin deposition. This apical fibronectin deposition was suggested to facilitate monocyte attachment directly through fibronectin interactions with the leukocyte integrin α4β1. However, it was later shown that α4β1 interactions with fibronectin play a minor role in leukocyte adhesion to the atherosclerotic endothelium compared with the canonical α4β1 ligand VCAM-1. We previously found that adhesion to a fibronectin matrix significantly enhances oxLDL-induced endothelial cell permeability, suggesting that fibronectin deposition may alter the cellular response to oxLDL. Therefore, we sought to determine whether matrix composition affects oxLDL-induced endothelial cell inflammatory response.

### Materials and Methods

Materials and Methods are available in the online-only Supplement.

### Results

Leukocyte interactions with the endothelium represent a critical and tightly regulated process in the inflammatory response. To assess the role of cell–matrix interactions in oxLDL-induced inflammation, we compared oxLDL-induced monocyte binding in human aortic endothelial cells plated on either basement membrane proteins or fibronectin. Although oxLDL treatment did not stimulate attachment of primary human monocytes or THP-1 monocytes in endothelial cells on basement membrane proteins (Figure 1A and Figure I in the online-only Data Supplement), endothelial cells on fibronectin showed a nearly 2-fold increase in monocyte attachment, suggesting that fibronectin augments oxLDL’s inflammatory properties.

Given the importance of endothelial ICAM-1 and VCAM-1 for monocyte adhesion, we next tested whether matrix composition affects their expression after oxLDL treatment. Treatment with 100 μg/mL oxLDL induced a rapid and transient increase in VCAM-1 mRNA (Figure 1B), whereas ICAM-1 mRNA expression was delayed but sustained (Figure 1C). Consistent with the monocyte adhesion data, endothelial cells on fibronectin show greater VCAM-1 and ICAM-1 expression at both the mRNA (Figure 1B and 1C) and protein (Figure 1D–1F) levels. Native LDL did not induce VCAM-1 expression, suggesting that these effects are specific to oxLDL (Figure II in the online-only Data Supplement). Importantly, the oxLDL used in these studies contained <10 pg/mL of endotoxin (Figure III in the online-only Data Supplement), which is 4 orders of magnitude below the threshold for endotoxin-induced ICAM-1/VCAM-1 expression (100 ng/mL; Figure III in the online-only Data Supplement). Although high concentrations of oxLDL can stimulate endothelial cell apoptosis, treatment with 100 μg/mL of oxLDL did not induce endothelial cell apoptosis as assessed by annexin V binding and analysis of cleaved caspase 3 and poly ADP ribose polymerase (Figure IV in the online-only Data Supplement).

The transcription factor nuclear factor-κB (NF-κB) regulates the expression of proinflammatory genes, such as ICAM-1 and VCAM-1. NF-κB-dependent transcription requires both nuclear translocation of the p65 subunit (hereafter referred to as NF-κB) and NF-κB phosphorylation on serine 536 in the transactivation domain. Therefore, we next tested whether matrix composition affects oxLDL-induced proinflammatory signaling by assessing NF-κB phosphorylation and nuclear translocation. Endothelial cells on fibronectin showed enhanced oxLDL-induced NF-κB phosphorylation (Figure 2A) and nuclear translocation (Figure 2B) compared with cells on basement membrane proteins. Consistent with NF-κB-driven expression, inhibiting NF-κB with either a pharmacological inhibitor (BAY 11-7082; Figure 2C) or a dominant-negative inhibitor of κB construct (super-repressor inhibitor of κB; Figure 2D) significantly blunted oxLDL-induced VCAM-1 expression. Taken together, these results suggest that matrix composition affects the ability of oxLDL to activate NF-κB-dependent transcription of proinflammatory genes.

Adhesion to fibronectin could enhance oxLDL’s proinflammatory response by altering the expression of oxLDL receptors, affecting oxLDL uptake, or modifying oxLDL-induced signaling, such as reactive oxygen species (ROS) production. However, fluorescence-activated cell sorting analysis of the endothelial oxLDL receptors oxLDL receptor 1, toll-like receptor 4, and CD36 showed no difference in the surface expression in response to matrix composition (Figure V in the online-only Data Supplement). Matrix composition also failed to affect uptake of Dil-labeled oxLDL (Figure V in the online-only Data Supplement). Because oxLDL-induced inflammation requires ROS in multiple cell types, we next tested for matrix-dependent ROS production after oxLDL treatment. Human aortic endothelial cells on either basement membrane or fibronectin were pretreated with 2′,7′-dichlorofluorescein diacetate, a redox-sensitive fluorogenic dye, and then treated with oxLDL. Like receptor expression and function, oxLDL-induced ROS production did not differ based on matrix composition (Figure V in the online-only Data Supplement).

Previous reports suggest that oxLDL can activate endothelial β1 integrins, presumably α5β1, the main fibronectin-binding integrin in endothelial cells. Because oxLDL-induced α5β1 integrin signaling could contribute to the altered NF-κB activation observed, we next tested whether oxLDL could stimulate α5β1 activation and α5β1-dependent signaling. To measure α5β1 activation, we used an α5β1-specific ligand mimetic consisting of a glutathione S-transferase fusion protein containing the 9th to 11th fibronectin type III repeats (GST-FNIII9–11) as previously described. Treatment with oxLDL enhanced GST-FNIII<sub>9–11</sub> retention (Figure 3A) without...
Figure 1. Matrix controls oxidized low-density lipoprotein (oxLDL)-induced inflammation. A, Human aortic endothelial cells (HAECs) on either basement membrane (BM) or fibronectin (FN) were treated with oxLDL (100 μg/mL) for 6 hours, and human primary monocyte adhesion was analyzed. Results are expressed as percent adherent to the endothelial monolayer. Representative images are shown (n=4). B and C, HAECs on different matrices were treated with oxLDL for the indicated times. mRNA was analyzed by quantitative real-time polymerase chain reaction for vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and GAPDH (n=4). D to F, VCAM-1, ICAM-1, and GAPDH protein expression was determined by Western blotting. Representative Western blots are shown (n=7). Values are means±SE. *P<0.05 compared with no treatment condition. #P<0.05 comparing matrices. NT indicates no treatment.
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affecting α5 surface expression (Figure 3B), consistent with enhanced α5β1 integrin activation. To test whether subsequent α5β1 ligation is required for oxLDL-induced VCAM-1 expression, we used blocking antibodies either to the integrin binding site in fibronectin (16G3) or to the α5β1 integrin (P1D6, SNAKA52). Blocking either fibronectin with 16G3 (Figure 3C) or the α5β1 integrin with P1D6 or SNAKA52 (Figure 3D) significantly blunted oxLDL-induced VCAM-1 expression. Control antibodies that bind to fibronectin but do not inhibit integrin binding (11E5) or that bind to other fibronectin-binding integrins such as αvβ3 (LM609) and αvβ5 (P1F6) did not affect oxLDL-induced VCAM-1 expression. Consistent with these findings, the α5-blocking antibodies also prevented oxLDL-induced NF-κB activation, whereas αvβ3 and αvβ5 blocking antibodies did not (Figure 3E). To verify the significance of integrin α5 in oxLDL-induced

Figure 2. Matrix regulates vascular cell adhesion molecule-1 (VCAM-1) expression through nuclear factor-κB (NF-κB). A, Human aortic endothelial cells (HAECs) plated on basement membrane (BM) or fibronectin (FN) were treated with oxidized low-density lipoprotein (oxLDL; 100 μg/mL) for the indicated times. Immunoblotting was performed for phospho–NF-κB (p–NF-κB; p65, Ser536) and GAPDH. Representative Western blots are shown (n=5). B, HAECs on different matrices were treated with oxLDL for 1 hour, and NF-κB nuclear translocation was determined by immunocytochemistry. Representative images are shown (n=3). C, HAECs were treated with Bay11-7082 (10 μmol/L; 1 hour), and oxLDL-induced VCAM-1 expression was analyzed. Representative Western blots are shown (n=3). D, OxLDL-induced VCAM-1 expression was determined in HAECs infected with either a cytomegalovirus an empty cytomegalovirus promoter (CMV) or a CMV-driven super-repressor inhibitor of κB (SR-IκBα)–expressing adenovirus. Representative Western blots are shown (n=3). Values are means±SE. *P<0.05 compared with no treatment condition. #P<0.05 comparing matrices. NT indicates no treatment.
Figure 3. Integrin α5 integrates oxidized low-density lipoprotein (oxLDL)-induced vascular cell adhesion molecule-1 (VCAM-1) and nuclear factor-κB (NF-κB) activation. A, Human aortic endothelial cells (HAECs) were treated with oxLDL (100 μg/mL) for 30 minutes, and α5β1 activation was determined by measuring glutathione S-transferase fusion protein containing the 9th to 11th fibronectin (FN) type III repeats (GST-FNIII9–11) retention by Western blotting. Representative images are shown (n=4). B, α5 surface expression in HAECs treated with oxLDL was determined by fluorescence-activated cell sorting analysis and is expressed as average mean fluorescence intensity (n=3). C, HAECs pretreated with the nonblocking (11E5, 40 μg/mL) or fibronectin-blocking antibody (16G3, 40 μg/mL) for 1 hour were treated with oxLDL for 6 hours, and VCAM-1 expression was determined by Western blotting (n=4). D and E, HAECs plated on FN were treated with blocking antibodies for α5 (P1D6, SNAKA52), αvβ3 (LM609), or αvβ5 (P1F6) at 10 μg/mL for 1 hour, and oxLDL-induced VCAM-1 expression (6 hours) and NF-κB activation (1 hour) were determined by Western blotting (n=4). F, OxLDL-induced VCAM-1 expression was determined in conditionally immortalized mouse aortic endothelial cells either wild type (empty cytomegolovirus promoter [CMV]) or deficient for α5 integrins (CMV-driven Cre recombinase expression [CRE]). Representative Western blots for VCAM-1, integrin α5, integrin αV, and extracellular signal–regulated kinase (ERK) are shown (n=3). Values are means±SE. *P<0.05 compared with no treatment condition. #P<0.05 compared with α5-deficient (Cre) cells. NT indicates no treatment.
**Figure 4.** Focal adhesion kinase (FAK) regulates oxidized low-density lipoprotein (oxLDL)–induced vascular cell adhesion molecule-1 (VCAM-1) and nuclear factor-κB (NF-κB) activation. 

- **A**, Human aortic endothelial cells (HAECs) were treated with oxLDL (100 μg/mL) for the indicated times. Immunoblotting was performed for phospho-FAK Y397, Y576, Y577, total FAK, and extracellular signal–regulated kinase (ERK). Representative images are shown (n=4).

- **B**, HAECs pretreated with blocking antibodies to α5 (P1D6, SNAKA52), αvβ3 (LM609), or αvβ5 (P1F6) were treated with oxLDL for 1 hour. Immunoblotting was performed for phospho-FAK Y397 and ERK. Representative images are shown (n=3).

- **C**, HAECs were pretreated with PF-573228 (10 μmol/L; 1 hour) and treated with oxLDL for indicated times. Immunoblotting was performed for phospho-FAK Y397, phospho–NF-κB (P–NF-κB), and ERK (n=4).

- **D**, HAECs pretreated with PF-573228 were treated with oxLDL for 6 hours, and VCAM-1 expression was determined by Western blotting. Representative images are shown (n=3).

- **E**, HAECs were transfected with anti-FAK small interfering RNA (siRNA) and treated for the indicated times. Immunoblotting was performed for FAK, P–NF-κB, and β-tubulin. Representative images are shown (n=4).

- **F**, HAECs were transfected with anti-FAK siRNA and then treated with oxLDL for 6 hours. Immunoblotting was performed for FAK, VCAM-1, and ERK. Representative images are shown (n=4). Values are means±SE. *P<0.05 compared with no treatment condition. #P<0.05 comparing treatment conditions. NT indicates no treatment.
inflammation, we isolated mouse aortic endothelial cells from mice expressing a floxed α5 integrin allele (gift of Richard Hynes, Massachusetts Institute of Technology). After Cre-mediated excision of the α5 gene, oxLDL-induced VCAM-1 expression was significantly reduced (Figure 3F). Together, these data suggest that α5β1 integrin activation plays a critical role in oxLDL-induced inflammation.

Because our data demonstrate a crucial role for α5 in oxLDL-induced inflammation, we next investigated the role of focal adhesion kinase (FAK), a common integrin signaling partner.9 OxLDL stimulated a rapid increase in FAK phosphorylation at the autophosphorylation site (Tyr397) and in the kinase domain (Tyr576, Tyr577) required for full FAK kinase activity (Figure 4A). Consistent with the role of α5β1 activation, FAK stimulation by oxLDL was completely inhibited by the α5β1-blocking antibodies P1D6 and SNAKA52 but not by the αvβ3-blocking antibody LM609 and the αvβ5-blocking antibody P1F6 (Figure 4B). The ATP-competitive FAK inhibitor, PF-573228, significantly repressed oxLDL-induced NF-κB activation (Figure 4C), VCAM-1 expression (Figure 4D), and monocyte adhesion (Figure 6V in the online-only Data Supplement). Additionally, FAK-targeted small interfering RNA (≈90% knockdown) similarly blunted oxLDL-induced proinflammatory responses (Figure 4E and 4F). Together, these data demonstrate that FAK signaling couples α5β1 to the NF-κB pathway after oxLDL stimulation thereby supporting the hypothesis that integrin signaling mediates oxLDL-induced endothelial cell activation.

Immunohistochemical analysis of plaques from human patients and atherosclerosis-prone mice demonstrated enhanced integrin α5 expression in both the endothelial cell layer and the invading macrophage foam cells (Figure VII in the online-only Data Supplement). Similarly, quantitative real-time polymerase chain reaction showed enhanced α5 expression in the aortic arch of Western diet–fed ApoE knockout mice compared with chow-fed ApoE mice and C57Bl/6J mice (Figure VII in the online-only Data Supplement). Because α5 signaling contributes to oxLDL-induced inflammation, we next tested whether inhibiting α5 signaling in vivo was sufficient to reduce atherosclerosis in hypercholesterolemic mice. Male, 8- to 10-week-old ApoE−/− mice were fed a high-fat Western diet for 8 weeks to induce atherosclerosis. On initiation of Western-diet feeding, mice were treated with either saline or the α5 signaling inhibitor ATN-161, a peptide mimetic of the PHSRN sequence of fibronectin,20 for the entire 8-week feeding regimen. ATN-161 was administered intraperitoneally at 5 mg/kg 3 times a week, consistent with previously published efficacy reports in murine cancer models.21,22 Treatment with the ATN-161 peptide did not affect mouse weight or blood glucose, total cholesterol, high-density lipoprotein cholesterol, LDL cholesterol, or triglyceride levels (Figure VIII in the online-only Data Supplement). However, ATN-161 treatment significantly limited diet-induced atherosclerosis as demonstrated by Oil Red O staining of the aorta (Figure 5A and 5B). Analysis of plaque cross sections using MOVAT staining showed a ≈40% reduction in plaque size in the aortic root (Figure 5C and 5D) and a ≈60% reduction in the carotid sinus (Figure IX in the online-only Data Supplement). Consistent with a role for α5β1 in endothelial activation, ATN-161 treatment reduced plaque-associated VCAM-1 expression in both the carotid sinus and innominate arteries (Figure 5E and 5F; Figure X in the online-only Data Supplement).

Although deletion of plasma fibronectin was previously shown to reduce plaque size and macrophage content,7 the smooth muscle–rich fibrous cap was similarly ablated, suggesting that targeting this pathway could lead to the formation of vulnerable plaques prone to rupture. To assess whether α5 inhibitors similarly affect plaque composition, we analyzed macrophage (Mac2-positive) and smooth muscle (smooth muscle actin positive) content of these plaques in the aortic root (Figure 6A). The number of plaques per section of the aortic root was similar between saline- and ATN-161–treated groups (Figure 6B), suggesting that ATN-161 reduces plaque area by limiting plaque size. ATN-161 treatment reduced the Mac2-positive regions in the aortic root and carotid sinus by 65% and 75%, respectively, indicative of diminished macrophage levels (Figure 6C; Figure IX in the online-only Data Supplement). Although smooth muscle actin–positive smooth muscle staining showed a trend toward lower levels (Figure 6D), this effect was not statistically significant. Furthermore, the percent area of the plaque positive for smooth muscle actin (Figure 6E), the thickness of the fibrous caps when present (Figure 6F), and the percentage of plaques scored positive for smooth muscle actin–rich fibrous caps (Figure 6G) were all unaltered in the ATN-161–treated mice. These data demonstrate that integrin α5 signaling contributes to the development of atherosclerosis, and blocking integrin α5 function significantly reduces plaque size by diminishing macrophage levels without affecting early smooth muscle recruitment.

**Discussion**

Transition to a fibroblast-rich matrix occurs before inflammatory cell recruitment, and limiting fibroblast deposition in vivo inhibits atherosclerosis and neointimal hyperplasia.7,8,10 OxLDL accumulates early in atherosclerosis and promotes endothelial cell activation.15,23 We demonstrate here that composition of the endothelial matrix modulates oxLDL-induced NF-κB activation, NF-κB–dependent proinflammatory gene expression (ICAM-1, VCAM-1), and monocyte attachment. This matrix-dependent response to oxLDL does not result from altered surface expression of oxLDL receptors, oxLDL uptake, or changes in ROS production. Instead, oxLDL treatment activates the fibronectin-binding integrin α5β1, and preventing α5β1 signaling (blocking antibodies, knockout cells) abolishes oxLDL-induced NF-κB activation and VCAM-1 expression. Both mouse and human atherosclerotic plaques show α5 expression in the endothelium and macrophages, and treating Western diet–fed ApoE knockout mice with the α5β1 signaling inhibitor, ATN-161, significantly reduces VCAM-1 expression, macrophage content, and atherosclerotic plaque size. Together, these data demonstrate that α5β1 integrin signaling critically regulates oxLDL-induced proinflammatory responses in early arterogenesis.

Despite nearly 30 years of study, several controversies still remain concerning the role of oxLDL in endothelial activation, including the presentation of oxLDL to endothelial cells, the degree of LDL oxidation, and the mechanism of
Figure 5. Inhibiting integrin α5 signaling in vivo is sufficient to delay atherosclerosis. Eight-week-old apolipoprotein E–deficient mice were fed a high-fat Western diet for 8 weeks, during which mice were treated with either saline or ATN-161 (5 mg/kg) by intraperitoneal injection. A and B, Oil Red O staining of aortas was performed, and plaque area was analyzed as the percent Oil Red O–positive area. C and D, Plaque size in the aortic root was quantified following Russell-MOVAT pentachrome staining. Plaque area was calculated as the neointimal area inside the internal elastic lamina. E and F, Vascular cell adhesion molecule-1 (VCAM-1) expression in the carotid sinus and innominate artery was determined by immunofluorescence immunohistochemistry and expressed as the positive area in the vessel wall. Images were taken at ×20, with insets at ×10. Analysis was performed using NIS elements software. n=8–10 mice per group. Values are means±SE.
Figure 6. Inhibiting integrin α5 signaling in vivo reduces macrophage content without altering fibrous cap formation. A, Aortic roots from saline- and ATN-161–treated mice were stained by immunohistochemistry for Mac2 (macrophage marker, green), smooth muscle actin (smooth muscle cell marker, red), and 4′,6-diamidino-2-phenylindole (DAPI; blue). Representative ×40 images are shown. B, The number of individual plaques per aortic root were quantified for each mouse. C, Macrophage area was analyzed by quantifying the Mac2-positive area for each aortic root. D, Smooth muscle area was analyzed by quantifying the smooth muscle actin (SMA)–positive area for each aortic root. E, The percentage of the plaque that was SMA positive was calculated by dividing the SMA-positive area by the total plaque area and averaged for each group. F, Thickness of the individual fibrous caps (averaged from >4 regions per cap) was calculated and expressed as the average cap thickness within each group. G, Plaques were scored for SMA-positive fibrous caps, and the percentage of fibrous cap–positive plaques was calculated by dividing by the total number of plaques in each group. Analysis was performed using NIS elements software. n=10 mice per group. Values are means±SE.
oxLDL-induced inflammation. The predominant theory on LDL oxidation suggests that LDL becomes oxidized after accumulation in the intima.\textsuperscript{24} Although this model might limit the potential for endothelial interaction with oxLDL, oxidation of the apoB\textsubscript{100} protein on LDL reduces its affinity for matrix, suggesting that it could diffuse to the endothelial surface.\textsuperscript{25} Additionally, several recent studies have found a pool of electronegative LDL similar to highly oxLDL, termed L5, in human plasma.\textsuperscript{26,27} Early studies identified minimally modified LDL, but not highly oxLDL, as the predominant inflammatory species\textsuperscript{28} and showed an important role for the oxidized phospholipid oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (oxPAPC) in mediating this effect.\textsuperscript{28,29} These studies did not observe ICAM-1 or VCAM-1 expression by oxLDL, minimally modified LDL (MM-LDL), or oxPAPC. However, other groups have shown oxLDL-induced ICAM-1 and VCAM-1 expression in arterial endothelial cells.\textsuperscript{31,32} However, highly oxLDL promotes NF-\kappa B activation in matrix-specific VCAM-1 expression, ATN-161 has only been shown to inhibit \( \alpha \beta_1 \) integrin signaling.\textsuperscript{43-45} Although these data are consistent with a role for \( \alpha_5 \) in endothelial cell activation, ATN-161 treatment may limit plaque formation through effects on other cell types as well. Inhibiting \( \alpha_5 \beta_1 \) in plaque macrophages could influence macrophage migration,\textsuperscript{46} and gene expression.\textsuperscript{48} Future studies examining cell type–specific deletion of \( \alpha_5 \) integrins should shed further light into the cellular mechanisms underlying this therapeutic effect.

Vascular fibronectin deposition affects leukocyte recruitment in multiple ways. Published work and data presented herein show that fibronectin deposition into the subendothelial matrix affects the endothelial cell activation response to multiple stimuli.\textsuperscript{7,8,10} However, apical deposition of fibronectin containing the alternatively spliced connecting segment (connecting segment 1) domain provides direct interaction sites for \( \alpha_4 \beta_1 \) integrins on leukocytes.\textsuperscript{13} Although MM-LDL stimulates apical fibronectin deposition, subsequent ex vivo analysis demonstrate that only a minority (<25%) of monocyte interactions with atherosclerotic endothelial cell occur through apical connecting segment 1 fibronectin.\textsuperscript{14} Interestingly, MM-LDL–induced apical fibronectin deposition is postulated to occur through \( \alpha_5 \beta_1 \) integrin activation, because MM-LDL enhances \( \beta_1 \) integrin ligation and blocking \( \alpha_5 \beta_1 \) prevents monocyte binding to MM-LDL–stimulated endothelial cells.\textsuperscript{13} However, maximal induction of monocyte binding with MM-LDL takes 4 hours,\textsuperscript{13,28} whereas \( \alpha_5 \beta_1 \) activation occurs rapidly (Figure 3A). Furthermore, cycloheximide treatment inhibits MM-LDL–induced monocyte binding, suggesting that new gene expression is required,\textsuperscript{28} and MM-LDL does not affect \( \alpha_5 \beta_1 \) expression.\textsuperscript{13} While data presented herein provide direct proof that oxLDL induces \( \alpha_5 \beta_1 \) activation, our work suggests that \( \alpha_5 \beta_1 \)-dependent signaling after interaction with subendothelial fibronectin supports oxLDL–induced proinflammatory gene expression.

Although the redox-sensitive transcription factor NF-\kappa B classically mediates proinflammatory ICAM-1 and VCAM-1 expression, there are conflicting reports as to NF-\kappa B’s role in oxLDL–induced endothelial inflammatory gene expression.\textsuperscript{30,33} Although macrophages stimulated with MM-LDL show enhanced NF-\kappa B signaling, MM-LDL fails to activate NF-\kappa B in endothelial cells.\textsuperscript{31,32} However, highly oxLDL promotes NF-\kappa B activation in endothelial cells, smooth muscle cells, and fibroblasts,\textsuperscript{30} and our data support an important role for NF-\kappa B activation in matrix-specific VCAM-1 expression by oxLDL. Interestingly, oxLDL stimulates ROS production independent of matrix composition, suggesting that ROS production is insufficient to activate NF-\kappa B. In our system, oxLDL activates NF-\kappa B through an \( \alpha_5 \beta_1 \) integrin-dependent pathway requiring the tyrosine kinase FAK. Although multiple groups implicate FAK signaling in NF-\kappa B activation and proinflammatory gene expression,\textsuperscript{33,34} the mechanisms by which oxLDL and \( \alpha_5 \beta_1 \)-dependent FAK signaling converge to activate NF-\kappa B remain unknown.

Although the integrin \( \alpha_5 \beta_3 \) has received considerable attention in cardiovascular disease models,\textsuperscript{35} much less is known about \( \alpha_5 \beta_1 \) integrins. Analysis of mRNA isolated from human plaques and abdominal aortic aneurysms shows enhanced \( \epsilon_5 \) expression,\textsuperscript{36,37} whereas protein levels were shown to be enhanced after carotid injury and induced atherogenesis.\textsuperscript{38,39} Immunohistochemistry from mouse and human plaques suggests that \( \alpha_5 \beta_1 \) expression occurs predominantly in the endothelial layer and plaque macrophages (Figure VII in the online-only Data Supplement). Our data suggest that endothelial \( \alpha_5 \) integrins contribute to proinflammatory gene expression and monocyte recruitment. The \( \alpha_5 \) integrin inhibitor ATN-161, a derivative of the fibronectin synergy sequence known to bind specifically to \( \alpha_5 \) and \( \epsilon_5 \) significantly reduces VCAM-1 expression, macrophage content, and atherosclerotic plaque size in hypercholesterolemic mice. Although ATN-161 can interact with multiple integrin \( \beta \) subunits,\textsuperscript{41,42} ATN-161 has only been shown to inhibit \( \alpha_5 \beta_1 \) integrin signaling.\textsuperscript{43-45} Although these data are consistent with a role for \( \alpha_5 \) in endothelial cell activation, ATN-161 treatment may limit plaque formation through effects on other cell types as well. Inhibiting \( \alpha_5 \beta_1 \) in plaque macrophages could influence macrophage migration,\textsuperscript{46} phagocytosis,\textsuperscript{47} and gene expression.\textsuperscript{48} Future studies examining cell type–specific deletion of \( \alpha_5 \) integrins should shed further light into the cellular mechanisms underlying this therapeutic effect.

Matrix-specific integrin signaling may contribute to multiple processes during atherosclerotic plaque formation, including endothelial activation, fibrous cap formation, and plaque angiogenesis.\textsuperscript{35} Several published studies suggest that limiting fibronectin deposition blunts early inflammation in the atherosclerotic plaque.\textsuperscript{8,10} and work from our group has shown that fibronectin enhances endothelial activation in response to multiple atherogenic stimuli.\textsuperscript{7,13,49} While genetic deletion of plasma fibronectin reduces endothelial activation and plaque inflammation,\textsuperscript{4} fibronectin deletion similarly reduced fibrous cap formation, suggesting that depleting fibronectin could destabilize the plaque promoting plaque rupture.\textsuperscript{8} However, we show that inhibiting the fibronectin-binding integrin \( \alpha_5 \beta_1 \) similarly reduces plaque inflammation without affecting early smooth muscle recruitment (Figures 5 and 6), potentially because of an important role for other fibronectin-binding integrins in smooth muscle growth and migration. Although other groups have shown an important role for \( \alpha_5 \beta_1 \) in smooth muscle growth and migration in cell culture models,\textsuperscript{50} our current data suggest that \( \alpha_5 \beta_1 \) may show limited expression in the smooth muscle, at least at early stages of plaque development. Taken together, our data suggest that \( \alpha_5 \beta_1 \) integrin inhibitors, currently in clinical trials targeting cancer, could be redirected for use in atherosclerosis.
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A.P. Mazar owns patents on the ATN-161 compound. The other authors report no conflicts.

Disclosures

References

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Significance

Endothelial cell activation facilitates monocyte recruitment into forming atherosclerotic plaques, and fibronectin deposition into the endothelial matrix enhances endothelial activation. We now show that oxidized low-density lipoprotein, a classic proatherogenic factor, stimulates the fibronectin-binding receptor, integrin α5β1. We further demonstrate that inhibiting α5β1 reduces oxidized low-density lipoprotein–induced proinflammatory adhesion molecule (vascular cell adhesion molecule-1) expression in cell culture models. Furthermore, treating atherosclerosis-prone mice with an integrin α5 inhibitor reduces vascular cell adhesion molecule-1 expression, macrophage content, and plaque burden without perturbing the protective fibrous cap. These findings are of high scientific interest because integrin inhibitors targeting α5 currently in clinical trials for other pathologies should be considered as potential therapeutics for atherosclerosis.
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METHODS

**Endothelial Cell Culture, Transfections, and Infections** – HAE cells (Lonza) were purchased at passage 3 and maintained in MCDB 131 supplemented with 10% FBS, 2mM glutamine, 10 U/mL penicillin (GIBCO), 100 µg/mL streptomycin (GIBCO), 60 µg/mL heparin sodium, and bovine brain extract (24 µg/mL) and were used between passages 6-10. Mouse aortic endothelial cells (MAECs) were isolated from integrin α5(ffi) mice (gift of Richard Hynes, MIT) as previously described1. Briefly, aortic rings (3-5mM) were placed on polymerized Matrigel to induce endothelial sprouts. Sprouting cells were isolated, sorted for the endothelial marker CD105 using magnetic beads, and reversibly transformed using a retroviral temperature-sensitive large T-antigen. The α5 gene was deleted following adenoviral infection with GFP-Cre or GFP control viruses and sorting for GFP positive cells. The temperature-sensitive large T antigen allows MAEC expansion at 33°C with IFNγ, whereas moving cells to 37°C in the absence of IFNγ for >3 days abrogates large T antigen expression. 6-well plates were coated with basement membrane extract (1:50 dilution, Trevigen) or fibronectin (10 µg/mL) overnight at 4°C, and cells were plated under low serum conditions (0.5% FBS). HAECs at 75% confluency were transfected with FAK SMARTpool siRNA (50 nM) using Lipofectamine 2000 (Life Technologies) for 2.5 hours on two consecutive days resulting in >90% knockdown. HAECs at 75% confluency were infected with the super-repressor IκB construct at 20 MOI for 24 hours.

**LDL oxidation** – LDL (Intracel) was oxidized by dialysis in PBS containing 13.6 µM Cu2SO4 for 3 days followed with 50 µM EDTA. This consistently displayed a relative electrophoretic mobility between 2 and 3, indicative of highly oxidized LDL. oxLDL was
stored under N₂ gas and tested for endotoxin contamination using a chromogenic endotoxin quantification kit (Thermo Scientific).

**mRNA isolation/qRT-PCR** – mRNA isolated from tissue and cells was lysed in TRIzol (Life Technologies) per manufacturer’s instructions. cDNA was synthesized using the Biorad iScript DNA Synthesis Kit. qRT-PCR was performed using a Biorad iCycler and Sybr Green MasterMix. Primers (listed in **Supplemental Table I**) were designed with the online Primer3 software and validated by melt curve analysis and sequencing of the resulting PCR product. Results were normalized to the housekeeping gene GAPDH and expressed as a fold change using the 2^ΔΔCt method.

**Monocyte Adhesion Assay** – Monocyte adhesion was performed as previously described². Primary human monocytes and human THP-1 monocytes were labeled with Cell Tracker Green (Invitrogen) according to manufacturer’s protocol. HAEC monolayers were treated as described and 1.0 x 10⁶ monocytes were added per well of a 24 well plate (~5:1 ratio of monocytes to endothelial cells). Monocytes were allowed to attach for 15 minutes at 37°C in HBSS containing calcium and magnesium. Supernatant and two washes were collected as the non-attached fraction, and both adherent and non-adherent monocytes were then lysed in 100mM NaOH. Fluorescence at 488 nm was quantified using a FLUOstar Optima fluorescence plate reader, and results were normalized to un-bound fraction and expressed at percent adhesion.

**Immunoblotting and Immunocytochemistry** – Cell lysis and immunoblotting was performed as previously described³. Antibodies used included rabbit anti-NF-κB (p65 subunit, s536), rabbit anti-ICAM-1, rabbit anti-GAPDH, rabbit anti-FAK (Y397), rabbit
anti-FAK (Y576), rabbit anti-FAK (Y577), rabbit anti-IκBα (Cell Signaling), rabbit anti-integrin α5, rabbit anti-integrin αV, rabbit anti-ERK1/2, rabbit anti-VCAM-1, rabbit anti-cleaved caspase 3, rabbit anti-cleaved PARP, rabbit anti-FAK, and mouse anti-GST (Santa Cruz). Densitometry was performed using ImageJ software. For immunocytochemistry, cells were fixed in 4% formaldehyde, permeabilized with 0.1% Triton X-100, blocked with 1% denatured bovine serum albumin (BSA) containing 10% goat serum, and incubated with rabbit anti-NF-κB (p65 subunit) antibody overnight. Cells were washed in TBST and incubated with Alexa488-conjugated goat anti-rabbit secondary antibodies for 2 hours. Unbound secondary was removed by TBST wash and coverslips were mounted onto microslides using Fluoromount G. Stains were visualized on a Nikon Eclipse Ti inverted epifluorescence microscope equipped with a Photometrics CoolSNAP120 ES2 camera and the NIS Elements 3.00, SP5 imaging software. Cells were scored for nuclear NF-κB, and at least 100 cells were counted per condition for each experiment.

**Dil-oxLDL uptake and ROS production** – For Dil-oxLDL uptake, cells were plated and treated with Dil-oxLDL at 20 μg/mL. Cells were rinsed twice with warm 1X PBS and read on a FLUOstar fluorescence plate reader and corresponding images taken on an epifluorescent microscope. Alternatively, cells were treated the same but fixed in 4% formaldehyde and images were taken. HAECs were cultured in phenol red-free media then loaded with DCFDA for 45 minutes and treated with oxLDL for various times. Cells were then read on a FLUOstar plate reader and arbitrary units were quantified as fold changes.
**FACS Analysis and Apoptosis Assay:** Cells were removed from the surface using Accutase (Millipore) and fixed in 4% Formaldehyde for 30 minutes. They were then blocked in 1% denatured albumin for 30 minutes. Cells were spun and incubated 1 x $10^6$ cells/ml with FITC-LOX1, FITC-CD36, FITC-TLR4 (Abcam) for one hour and baseline was established with no antibody and IgG isotype control. Apoptosis analysis was performed using a commercially available kit based on Annexin V staining and flow cytometry (Life Technologies).

**Integrin Activation:** A glutathione S-transferase (GST) fusion protein corresponding to the 9th, 10th, and 11th type III repeats on fibronectin was used to measure $\alpha_5\beta_1$ activity as previously described. After stimulation with oxLDL, cells were incubated with 20 μg/ml of GST-FNIII9-11, in 1X PBS containing 1 mM Ca$^{2+}$/1 mM Mg$^{2+}$ at 37°C for 30 min. Cells were washed twice, and lysed in 2X Laemmli Buffer. Attached GST-FNIII9-11 was assessed by Western blotting for the GST tag.

**Animals and Tissue Harvest:** Animal protocols were approved by the LSU Health Sciences Center-Shreveport institutional animal care and use committee, and all animals were cared for according to the National Institute of Health guidelines for the care and use of laboratory animals. All experiments using human tissue was deemed non-human research by the local IRB due to the exclusive use of postmortem samples. Human tissue was excised postmortem during routine autopsy at the LSU Health Sciences Center-Shreveport and was classified according to the Stary system of atherosclerosis. Male ApoE$^{-/-}$ mice (8-10 weeks old) were placed on a high fat Western Diet (TD 88137 [Harlan-Teklad]; containing 21% fat by weight, 0.15% cholesterol by weight, and 19.5% casein by weight without sodium cholate) for 8 weeks before
euthanasia. Concomitant with the initiation of Western diet feeding, mice were treated with either saline or ATN-161 (5 mg/kg) by intraperitoneal injection three times a week for the duration of the experiment. Glucose and weight were monitored weekly using an AlphaTRAK Glucometer (Abbott). After 8 weeks on diet, mice were euthanized by pneumothorax under anesthesia, and blood was collected by cardiac puncture into heparinized blood collection tubes. Blood was centrifuged at 5000 rpm for 5 minutes, and plasma was isolated and frozen at -80°C until analysis could be performed. Following blood collection, mice were perfused with 4.0% formaldehyde, and atherosclerosis prone regions, including the aorta, aortic root, innominate artery, and carotid sinus were collected for immunohistochemistry.

**Plaque Analysis, Immunohistochemistry, and Blood Analysis:** Aortas were harvested, cleaned of adventitia, cut open longitudinally, pinned and stained with Oil Red O (Alfa Aesar). Vessels were visualized on a DS-Fi1 camera (Nikon) attached to a multizoom AZ100 microscope (Nikon), and plaque burden was analyzed using Nikon Elements software and expressed as a percentage of total aortic area. The aortic root, innominate artery, and carotid sinuses were processed for paraffin embedding and cut into 5 µm sections. All sections within each staining regimen were taken from the same site at equal distance from anatomical landmarks (ie. carotid bifurcation, initiation of valve leaflets, brachiocephalic branchpoint). Immunohistochemistry was performed as previously described. Antibodies included goat anti-CD31 (Santa Cruz), rat anti-Mac2 (Accurate Chemical), mouse anti-smooth muscle actin (Sigma), mouse anti-CD68 (Dako), mouse anti-vWF (Abcam), rabbit anti-integrin α5 (LSBio), and rabbit anti-VCAM-1 (Cell Signal). Staining was visualized either with Alexa-Fluor (Life Technologies)
conjugated or biotinylated secondary antibodies, the Vectastain ABC kit, and 3-3’-diaminobenzidine (DAB, Dako). The percentage of the vessel wall staining positive for fluorescence or DAB was determined using Nikon Elements software. Total vessel area did not change between treatments. We also determined plaque area by performing the Russell-Movat pentachrome stain and the vessel area inside the internal elastic lamina was quantified using Nikon Elements software. Total and HDL cholesterol (Wako) and triglyceride levels (Pointe Scientific) were measured using commercially available kits. LDL was calculated using the Friedewald equation.

**Statistical Analysis:** Statistical analysis was performed using GraphPad Prism software. Data was tested for Normality (Kolmogorov-Smirnov test). Data that passed the Normality assumption was analyzed using Student’s T-test, one-way ANOVA with Newman-Keuls post-test or two-way ANOVA with Bonferroni post-tests. Data that failed the Normality assumption were analyzed using the non-parametric Mann-Whitney U test and the Kruskal Wallis test with post hoc analysis. Error bars indicate standard error.

HAECS on either BM or FN were treated with oxLDL (100 μg/mL) for 6 hours and THP-1 monocyte adhesion was analyzed. Results are expressed as percent adherent to the endothelial monolayer. Representative images are shown, n=4. Values are means ± SE, *p < 0.05 compared with no treatment condition, #p < 0.05 comparing matrices.
Native LDL does not result in VCAM-1 expression. HAECs were plated on FN and treated with either LDL (100μg/mL) or oxLDL (100μg/mL) for 6hr then lysed for immunoblotting, n=3.
OxLDL preparations do not contain endotoxin. (A) Oxidized LDL was analyzed for endotoxin contamination after preparation by using an LAL chromogenic endotoxin kit. (B) HAECs were plated on FN and treated with different doses of lipopolysaccharide (1pg/ml-100μg/ml) for 6hr then lysed for immunoblotting, n=3.
Supplemental Figure IV

OxLDL does not elicit apoptosis. (A) Apoptosis was analyzed in HAECs plated on different matrices in response to oxLDL or camptothecin (used as a positive control) for 24 hours using a commercially available kit (Invitrogen), n=3. (B) HAECs were plated on different matrices and treated with oxLDL, immunoblotting for cleaved caspase 3 was analyzed by densitometry, n=3. (C) HAECs were plated on different matrices and treated with oxLDL, immunoblotting for cleaved PARP was analyzed by densitometry, n=3. (D) Representative images of cleaved caspase 3 and cleaved PARP are shown.
Matrix-dependent inflammation is independent of scavenger receptor expression, oxLDL uptake, and ROS. (A) FACS analysis was performed for oxLDL receptors on HAECs plated on either BM or FN, n=3. (B) Dil-oxLDL (10 μg/mL) uptake was determined by visualization on an epifluorescent microscope and expressed as a fold change in arbitrary fluorescence units, n=3. (C) OxLDL-induced reactive oxygen species production was determined in cells loaded with the redox-sensitive dye, H2-DCFDA. Results are expressed as a fold change in arbitrary fluorescence units, n=3. Values are means ± SE.
Figure 4. FAK mediates oxLDL-induced p65 nuclear translocation and monocyte adhesion. (A/B) HAECs pretreated with PF-573228 for 1 hour were then treated with oxLDL for 0, 30, 60, and 120 minutes. Cells were then fixed and immunostained for NF-κB (p65). Nuclear translocation was determined by immunocytochemistry and quantified. Representative images are shown, n=3. Values are means ± SE, *p < 0.05 compared with no treatment condition, #p < 0.05 comparing to inhibitor. (C/D) HAECS were pretreated with PF-573228 for 1 hour then treated with oxLDL (100 μg/mL) for 6 hours and THP-1 monocyte adhesion was analyzed. Results are expressed as fold increase compared to no treatment. Representative images are shown, n=4, *p < 0.05.
Figure 5. Integrin α5 is upregulated in human and murine atherosclerotic tissues. (A/B) Human vessels scored for atherosclerotic plaques were stained for α5 integrins (green), endothelium (white), and leukocytes (CD68) and counterstained with DAPI (blue). Representative micrographs of plaque-containing (A) and plaque-free (B) regions are shown. n = 10. (C) The carotid sinus isolated from ApoE knockout mice fed a Western Diet for 8 weeks was processed for immunohistochemistry and stained for α5 integrins (green), endothelium (CD31, white), and leukocytes (Mac2, red) and counterstained with DAPI (blue). Representative micrographs are shown. n = 4. (D) mRNA isolated from the aortic arch and thoracic aorta of C57Bl/6J mice, ApoE-/- mice fed a chow diet, and ApoE-/- mice on a high fat diet were analyzed for integrin alpha 5. 2Δct mRNA analysis was performed based on the ratio of aortic arch/thoracic aorta, n=5, ** p<0.01.
ATN-161 treatment does not alter weight gain, glucose, or metabolic panels. (A) Weight and (B) glucose were measured in 8 week old C57B6 (ApoE -/-) mice that were placed on a Western Diet treated with either saline or ATN-161 intraperitoneally. Black-Saline, Blue-ATN-161. (C) Quantitative analysis for total cholesterol, HDL, and triglycerides levels performed for mice previously described using commercially available kits. LDL was quantified by utilizing the Friedewald equation. n=10, values are means ± SE.
Inhibiting integrin α5 signaling in vivo reduces plaque size and macrophage content, in the carotid sinus. (A, B) Carotid sinuses of Saline and ATN-161 injected mice were analyzed by Russell-Movat pentachrome stain and plaque size quantified. (D, E) Carotid sinuses were stained for Mac2 (macrophage marker) and macrophage content was analyzed. Analysis was performed using NIS elements software, n=10. Serial sections were used and values are means ± SE, *p < 0.05 compared with saline treatment.
Supplemental Figure X

VCAM-1 expression in the innominate artery. The innominate artery of saline and ATN-161 injected mice were analyzed for VCAM-1 staining. Analysis was performed using NIS elements software, n=4-6.
### Supplemental Table I

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<th>Gene</th>
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