Role of Endothelial N-Glycan Mannose Residues in Monocyte Recruitment During Atherogenesis

David W. Scott, Jie Chen, Balu K. Chacko, James G. Traylor Jr, Anthony W. Orr, Rakesh P. Patel

Objective—Upregulated expression of endothelial adhesion molecules and subsequent binding to cognate monocyte receptors are established paradigms in atherosclerosis. However, these proteins are the scaffolds, with their posttranslational modification with sugars providing the actual ligands. We recently showed that tumor necrosis factor-α increased hypoglycosylated (mannose-rich) N-glycans on the endothelial surface. In the present study, our aim was to determine whether (1) hypoglycosylated N-glycans are upregulated by proatherogenic stimuli (oscillatory flow) in vitro and in vivo, and (2) mannose residues on hypoglycosylated endothelial N-glycans mediate monocyte rolling and adhesion.

Methods and Results—Staining with the mannose-specific lectins concanavalin A and lens culinaris agglutinin was increased in human aortic endothelial cells exposed to oscillatory shear or tumor necrosis factor-α and at sites of plaque development and progression in both mice and human vessels. Increasing surface N-linked mannose by inhibiting N-glycan processing potentiated monocyte adhesion under flow during tumor necrosis factor-α stimulation. Conversely, enzymatic removal of high-mannose N-glycans, or masking mannose residues with lectins, significantly decreased monocyte adhesion under flow. These effects occurred without altering induced expression of adhesion molecule proteins.

Conclusion—Hypoglycosylated (high mannose) N-glycans are present on the endothelial cell surface at sites of early human lesion development and are novel effectors of monocyte adhesion during atherogenesis. (Arterioscler Thromb Vasc Biol. 2012;32:e51-e59.)

Key Words: adhesion ■ glycobiology ■ inflammation ■ atherosclerosis ■ vascular

Atherosclerosis is a progressive inflammatory disease of the artery walls, which leads to the formation of vascular plaques that can occlude blood flow or rupture, leading to stroke or myocardial infarction.1,2 Early progression of the disease is characterized by infiltration of leukocytes, particularly monocytes, into the inflamed tissue.3 To exit the circulation, monocytes follow a cascade of tethering, rolling, adhesion, and finally transmigration through endothelial cells (ECs) lining the vessel wall.4 A common paradigm for interactions between ECs and monocytes suggests that proinflammatory signals induce expression of adhesion molecules on the EC surface, which directly interact with monocyte receptors leading to their rolling and adhesion.4,4 Interestingly, nearly all plasma membrane and secreted proteins are N-glycosylated. N-glycosylation is the co-/posttranslational addition of oligosaccharides (glycans) onto the amide group of asparagine residues in an N-X-S/T motif.7 Protein N-glycosylation occurs via a multistep and sequential process, resulting in high-mannose, hybrid, and then complex N-glycans. The latter are the most diverse and branched family of N-glycans, and it is thought that under basal conditions, most surface glycoproteins exist in this fully processed form, with hypoglycosylated N-glycans (high mannose/hybrid N-glycans) remaining at low levels.8 N-glycans are located at the interface between the endothelium and the circulation, positioning them for possible interactions with monocytes. In this manner, the protein adhesion molecules act as scaffolds for the N-glycans, which serves as the actual ligands for monocytes, a concept that has been discussed previously.9,10 In this paradigm, adhesion molecules alone are not sufficient to mediate adhesion, but require the appropriate complement of carbohydrate moieties to function, forming a molecular zip code for immune cell recognition.11 Indeed, a role for correct glycosylation of proteins as ligands of immune cells is well accepted. For example, proper N-glycosylation is required for CD44 to be recognized as a selectin ligand.12 At this time, little is known with regard to the role of endothelial mannose residues in inflammation and immune cell recruitment. It should be noted, however, that immune cells express various mannose-specific receptors to mediate substrate recognition and signaling, and some of these receptors have been implicated in atherosclerosis.13,14 Recently, Matthijsen et al15 showed that macrophage-expressed mannose-binding lectin (MBL) enriched in early atherosclerotic lesions and dendritic cell–specific intercellular adhesion molecule-3-grabbing nonintegrin, which participates in cell adhesion via binding
of high-mannose structures, has also been found in early lesions.\textsuperscript{16,17}

Recently, we demonstrated that tumor necrosis factor-\textalpha (TNF-\textalpha) treatment increases hypoglycosylated, high-mannose, and hybrid N-glycans on the EC surface.\textsuperscript{18} In vivo, atherosclerotic plaque progression is triggered by a variety of proinflammatory signals, including TNF-\textalpha, but a persistent pathogenic trigger of early EC dysfunction is the complex blood flow profiles at branch points, curvatures, and bifurcations in arterial vessels.\textsuperscript{19} Herein, we expand on our previous findings and demonstrate that disturbed flow increases hypoglycosylated N-glycans and that these glycans are also enriched in early human atherosclerotic plaques. Tissue culture models demonstrate that inhibiting N-glycan maturation increases monocyte adhesion, but only under conditions of flow. Finally, enzymatic removal of high-mannose N-glycans or masking mannose residues with targeted lectins attenuates monocytes adhesion to TNF-\textalpha–stimulated cells. Collectively, these data identify endothelial mannose as a novel ligand for monocyte adhesion during atherogenesis.

Materials and Methods

Detailed materials and methods are found in the in the online-only Data Supplement. Detection of high mannose/hybrid N-glycans was determined by staining stimulated human aortic ECs (HAECs) with the fluorescein isothiocyanate–conjugated mannose–specific lectins concanavalin A (ConA) and lens culinaris agglutinin (LCA).\textsuperscript{20} The specificity of these lectins for mannose on tissue sections or cultured cells was demonstrated by loss of staining in the presence of \textalpha-methylmannose (500 mmol/L) as described\textsuperscript{18} and as shown in Figure I in the online-only Data Supplement. In vivo detection of mannose residues was determined by ConA and LCA staining of murine and human vessel sections at various stages of lesion progression. All animal protocols were approved by the Louisiana State University Health Sciences Center-Shreveport institutional animal care and use committee according to National Institutes of Health guidelines. Experiments with human tissue were considered nonhuman because of the use of exclusively postmortem samples. Adhesion molecule expression in HAECs was monitored by Western blot analysis, surface ELISA, and immunocytochemistry. Rolling and adhesion of human acute monocytic leukemia cell line (THP-1) monocytes and primary human monocytes were determined using the Glycotech flow chamber as described.\textsuperscript{18}

Results

Increase in Hypoglycosylated N-Glycans in Response to Atheroprone Flow and at Sites of Plaque Development In Vivo

Our previous work demonstrated that TNF-\textalpha stimulation increased hypoglycosylated N-glycans on the surface of ECs.\textsuperscript{18} Although signaling through TNF-\textalpha and other proinflammatory cytokines induces endothelial dysfunction, a persistent pathogenic trigger in vivo for atherogenesis is the complex shear profiles in regions of disturbed flow.\textsuperscript{19} To determine whether atheroprone shear also increases hypoglycosylated N-glycans, ECs were kept static or exposed to either atheroprotective laminar shear stress or atheroprone oscillatory shear stress for 18 hours. Oscillatory shear stress induced increased levels of hypoglycosylated N-glycans as indicated by increased binding of the mannose–specific lectins ConA and LCA compared with either static or laminar shear stress conditions (Figure 1A and 1B; Figure II in the online-only Data Supplement).

In vivo atherosclerotic lesion progression occurs in vessels subjected to disturbed flow, such as the innominate artery and the portion of the right common carotid before the brachiocephalic branch point. To determine whether atheroprone regions of the vasculature express increased hypoglycosylated N-glycans, we examined the innominate artery of 16-week-old apolipoprotein E–deficient mice that had been maintained on a chow diet. At this age, early atherosclerotic plaque formation in the innominate artery has not developed past the early fatty streak stage of plaque progression. As seen in Figure 1C, healthy (no lesion) regions of the vasculature did not show luminal staining with rhodamine ConA. In contrast, early lesions expressed strong reactivity toward ConA, demonstrating that hypoglycosylated N-glycans are present at sites of monocyte adhesion in vivo. Figure 1C also shows staining of platelet EC adhesion molecule-1 on adjacent sections, illustrating

![Image](http://atvb.ahajournals.org/)

**Figure 1.** Increase in concanavalin A (ConA) and lens culinaris agglutinin (LCA) staining by oscillatory flow and in regions of early plaque development. Human aortic endothelial cells were left static or exposed to laminar shear stress (LSS) or oscillatory shear stress (OSS) for 18 hours before being labeled with fluorescein isothiocyanate–conjugated LCA and ConA. Representative images are shown in A and quantification in B. n=4; *\textless P<0.05 compared with static control and #\textless P<0.05 compared with LSS by 1-way ANOVA with Tukey posttest. C. Apolipoprotein E–deficient (apoE\textsuperscript{−/−}) mice were fed a standard chow diet for 16 weeks. The innominate artery was removed stained with ConA or antiplatelet endothelial cell adhesion molecule-1 (PECAM-1). Shown are representative images from serial sections from the same vessel. Photos from areas of the vessel where early stages of a lesion were visible are indicated by the arrow. Endothelial cell surface ConA reactivity was only observed on lesions. Red, rhodamine conjugated ConA; green, autofluorescence of basement membrane; blue, nuclei. n=4 mice.
that ConA-reactive epitopes are present on the endothelium. These data demonstrate that hypoglycosylated N-glycans are positioned in the appropriate regions of the vasculature and expressed at the appropriate time of lesion development to act as mediators of monocyte adhesion.

We next determined whether human atherosclerotic lesions contain hypoglycosylated N-glycans. A total of 24 vessels from 18 patients ranging in Stary score from 1 to 52,22 from the previously described collection were analyzed23 (described in Tables I and II in the online-only Data Supplement). Under this scoring system, stage 1 corresponds to the first appearance of monocyte-derived macrophages in the intima, stage 2 corresponds to fatty streak formation and significant accumulation of lipid-filled foam cells, and stage 3 refers to an early fatty streak lesion with extracellular lipid pools. Stage 4 plaques and above are recognized as advanced lesions containing a core of extracellular lipid and fibrotic changes and representing clinically symptomatic disease. When stained with rhodamine ConA and LCA, luminal regions of the vasculature showed low reactivity in plaque-free and stage 1 lesions (Figure 2A–2D). Reactivity toward ConA and LCA dramatically increased in stage 2 and 3 lesions and, importantly, was localized to the luminal surface where they would encounter monocytes (Figure 2E–2H). Interestingly, staining became variable in advanced stage 4 and 5 lesions (Figure 2I–2J), with 5 of 9 sections examined showing strong luminal reactivity with ConA and LCA. Staining specificity was demonstrated by the ability to block ConA and LCA by including reactivity with ConA and LCA. Staining specificity was demonstrated by the ability to block ConA and LCA by including antigens stained: no plaque, n=1 (A, B); stage 1, n=2 (C, D); stage 2, n=7 (E, F); stage 3, n=6 (G, H); stage 4/5, n=9 (I, J).

Figure 2. Increased hypoglycosylated N-glycans at site of early atherogenesis in human vessels. Serial sections of human coronary and carotid artery scored by Stary classification were stained with rhodamine concanavalin A (ConA) and lens culinaris agglutinin (LCA). Red, lectin; green, autofluorescence of basement membrane; blue, nuclei. Images are representative of vessels stained: no plaque, n=1 (A, B); stage 1, n=2 (C, D); stage 2, n=7 (E, F); stage 3, n=6 (G, H); stage 4/5, n=9 (I, J).

These results show that hypoglycosylated N-glycans are present at the luminal surface during early atherosclerotic progression.

α-Mannosidase Inhibitors Increase Surface Mannose Content and Potentiate Monocyte Rolling and Adhesion

With our findings that hypoglycosylated N-glycans are present during early lesion formation, we next investigated whether these structures could provide monocyte-binding epitopes. Several studies suggest that hypoglycosylated glycoproteins are defective in trafficking to the cell surface. To determine whether adhesion molecules generated in the presence of N-glycan inhibitors could reach the cell surface, specific steps in the N-glycan processing were inhibited, and surface expression and distribution of candidate adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were analyzed. Inhibitors used were tunicamycin that blocks transfer of N-glycans by inhibiting oligosaccharyltransferase, resulting in nonglycosylated proteins, kifunensine (kif), an α-mannosidase class 1 enzyme inhibitor, resulting in only high-mannose N-glycans being produced, and swainsonine (swain), an α-mannosidase class 2 inhibitor, resulting in high-mannose and minimally processed hybrid N-glycans. HAECS were pretreated with inhibitors before stimulation with TNF-α. As seen in Figure 3A, each enzyme effectively inhibited the processing of glycoproteins as indicated by the molecular weight (MWt) shifts in ICAM-1 and VCAM-1 by Western blot analysis. Importantly, complete inhibition of each enzyme was achieved as indicated by the appearance of only 1 band (different glycoforms would run at different MWts because of the diversity of carbohydrate content). Tunicamycin decreased ICAM-1 and VCAM-1 MWt to ≈52 and 76 kDa (corresponding to predicted MWts from primary sequences), indicating that N-glycans constitute ≈45% and 15% of the TNF-α-stimulated proteins, respectively. Interestingly, kif and swain had similar effects on the decrease in MWt of VCAM-1, but resulted in the production of distinct ICAM-1 glycoforms, with larger glycoforms being seen for swain, consistent with the prediction that kif would result in smaller high-mannose N-glycans and swain would allow for processing to the hybrid stage. Next, to determine whether hypoglycosylated adhesion molecules reach the apical membrane, surface ELISAs were performed on cells treated with inhibitors, TNF-α, or a combination of the 2 (Figure 3C and 3D). Neither kif nor swain altered the surface expression levels of ICAM-1 or VCAM-1, but tunicamycin blocked their transport to the cell surface. To test whether kif and swain altered the surface distribution of adhesion molecules, staining of ICAM-1 and VCAM-1 in unpermeabilized TNF-α-stimulated HAECS was performed. Surface staining patterns were similar between normal and hypoglycosylated ICAM-1 and VCAM-1 (Figure 3B). Finally, to confirm that the inhibitors were altering the monosaccharide content on the EC surface, cells exposed to inhibitors or in combination with TNF-α were stained for the presence of surface ConA- and LCA-reactive carbohydrates. As seen in Figure 3E, both kif and swain increased the surface levels of ConA and, in combination with TNF-α, further increased ConA levels above TNF-α treatment alone. Similar
results were observed with LCA (Figure 3F), except that kif did not enhance TNF-α–dependent effects. As expected, cells pretreated with tunicamycin did not show an increase in ConA or LCA with TNF-α stimulation and also completely inhibited ICAM-1, VCAM-1 trafficking to the plasma membrane (Figure 3C and 3D). These data demonstrate that α-mannosidase inhibitors kif and swain effectively increase surface mannose content, restricting N-glycan processing to the complex types, without compromising the ability of adhesion molecules to reach the EC cell surface.

Endothelial Hypoglycosylated N-Glycans Regulate Monocyte Adhesion Under Flow

We next wanted to determine whether hypoglycosylation of adhesion molecules on the EC surface would affect monocyte adhesion. ECs were left untreated, TNF-α treated, or treated with α-mannosidase inhibitors alone or in combination with TNF-α. Under static conditions, TNF-α increased THP-1 and primary human monocyte adhesion, and this was not altered by pretreatment with kif or swain (Figure 4A and 4B). However, in the presence of flow (0.5 dyne/cm²), kif and swain potentiated the number of both THP-1 and primary human monocytes adhering to TNF-α–stimulated ECs (Figure 4C and 4D). Neither kif nor swain alone affected THP-1 or primary human monocyte adhesion relative to control. These data demonstrate that adhesion molecule hypoglycosylation increases TNF-α–dependent monocyte adhesion. Figure III in the online-only Data Supplement shows that kif and swain alone increase the number of rolling monocytes compared with control, but not to the degree of TNF-α. This suggests that rolling of monocytes can be mediated, at least in part, by hypoglycosylation of constitutive proteins, whose presence on the endothelial surface is not absolutely dependent on TNF-α activation. However, this rolling is not transmitted to increased firm adhesion as indicated in Figure 4D.

Removal or Blocking of Mannose Residues Attenuates Monocyte Adhesion

To confirm a role for EC hypoglycosylated, high-mannose N-glycans on EC–monocyte adhesion, cells were treated with TNF-α and fixed before being exposed to the high-mannose
N-glycan-specific endoglycosidase H. Figure 5A shows that endoglycosidase H treatment of cells resulted in ~70% decrease in ConA staining. No change in the staining levels of the complex N-glycan lectin, Sambucus nigra agglutinin was observed (Figure 5A). In addition, endoglycosidase H digestion did not change the expression of ICAM-1 nor E-selectin on the cell surface (Figure 5A), thereby leaving the protein scaffolds for N-glycans unchanged. We next examined the effects of endoglycosidase H digestion on monocyte adhesion. Removing high-mannose N-glycans from the surface of ECs did not alter monocyte adhesion under static conditions (Figure 5B) but resulted in ~60% reduction in the number of adherent monocytes under flow (Figure 5C).

To further test whether endothelial mannose residues were participating in monocyte adhesion, a lectin blocking experiment was performed using 3 mannose-specific lectins. HAECs were treated with TNF-α and then exposed to ConA, LCA, or the α-1,3 mannose-specific lectin galanthus nava- lis lectin for the last 10 minutes of TNF-α incubation. Cells were then washed and monocyte adhesion measured. This approach would allow for masking of mannose residues on the cell surface and block any potential interactions with hypoglycosylated N-glycan recognition receptors on monocytes. As seen in Figure 6A, blocking surface mannose residues with lectins did not change monocyte adhesion under static conditions. However, as seen in Figure 6B, in the presence of flow both ConA and galanthus nava- lis lectin significantly reduced, whereas LCA modestly reduced, monocyte adhesion. The ability of lectins to inhibit monocyte adhesion was dose-dependent (Figure IV in the online-only Data Supplement). Collectively, these data show that the expression of the adhesion molecule protein scaffold is necessary, whereas their modification with N-glycan epitopes is conditional, but required for monocyte adhesion under flow.

**Discussion**

The work presented herein represents a new paradigm for monocyte–EC interactions, where hypoglycosylated high-mannose and hybrid N-glycans on adhesion molecules act as ligands for monocyte rolling and adhesion. Atheroprone shear and sites of early atherosclerotic lesions in murine and human tissues show elevated levels of mannose-specific lectin staining (Figures 1 and 2). Induced hypoglycosylation does not alter TNF-α–induced adhesion molecule trafficking or distribution (Figure 3), but does exacerbate monocyte adhesion (Figure 4), which can be attenuated by removing high-mannose N-glycans or masking mannose residues (Figures 5 and 6). Collectively, these data identify endothelial mannose as a novel ligand and regulator of monocyte adhesion under flow.

Protein N-glycosylation is a multistep pathway, whereby oligosaccharides are enzymatically added to asparagine residues in N-X-S/T motifs. A sequential process of carbohydrate trimming and addition gives rise to the 3 subtypes of N-glycans: high mannose (5–9 mannose), hybrid (3–5 mannose), and complex (3 mannose). It is thought that most N-glycans are processed to the complex stage, which helps maintain their surface localization by interaction with the galectin lattice. However, our previous data and the current findings suggest that proatherogenic stimuli increase hypoglycosylated N-glycan content on the EC surface. These findings are supported by work showing that proinflammatory stimuli modifies glycan profiles on synoviocytes and ECs. Cancer, which has a recognized proinflammatory component, is associated with an increase in high-mannose N-glycans.
and loss of N-glycan complexity of E-cadherin is associated with cancer progression.28 Also, hypoglycosylated N-glycans on various cell types have been implicated in the adhesion of neutrophils,29–31 demonstrating that they can be maintained on the surface of cells. Collectively, these data suggest that N-glycan processing is regulated during inflammatory stress.

Our current data extend these concepts to include EC–monocyte interactions in atherosclerosis development. We demonstrate that atheroprone oscillatory flow, which is an established stimulus to increase adhesion molecule expression,32–34 also increases expression of hypoglycosylated N-glycans on the surface of ECs. Of critical importance, early sites of plaque development in vivo, which represent focal regions of inflammation and monocyte adhesion, were highly decorated by hypoglycosylated N-glycans. The exact mechanism underlying hypoglycosylation of these N-glycans is unclear. One possible mechanism involves TNF-α–dependent downregulation of α-mannosidase activity (which trims mannose residues from high-mannose structures, thereby providing substrate for the formation of complex N-glycans).18 This is also suggested by recent findings from array studies showing upregulated mannosidase α class 1A member 1 in EC exposed to laminar shear stress compared with oscillatory shear stress.
which supports the concept that anti-inflammatory laminar shear stress promotes complex N-glycan production. A decrease in α-mannosidase activity has also been reported in rat aortas during drug-induced diabetes mellitus, which can be returned to normal levels with insulin administration, suggesting that inflammation is the underlying cause of the decrease in enzyme activity. Diabetes mellitus represents a significant risk factor for endothelial dysfunction and atherosclerosis, and dysregulation of N-glycan processing as an underlying modulator of disease demands future investigations.

Our data show that proinflammatory stimuli increase mannose residues on the EC surface via enrichment of hypoglycosylated N-glycans. Immune cells express a variety of receptors that can bind to mannose, including some which prefer hypoglycosylated N-glycans. If immune cells can target hypoglycosylated N-glycans, then a role of these N-glycans in autoimmune diseases would be expected. Indeed, multiple sclerosis, systemic lupus erythematosus, and congenital dyserythropoietic anemia are all associated with hypoglycosylated N-glycans. In this context, dysfunction in N-glycan processing has been postulated as a danger signal that can initiate immune responses because many primitive organisms, such as viruses, fungi, and bacteria, express high levels of mannose-rich glycan on their surfaces. The appearance of mannose-rich N-glycans on endogenous proteins could be viewed as nonself and illicit an immune response. We note that increased ConA or LCA staining was not restricted to the endothelial monolayer in mouse or human atherosclerotic lesions, and although less intense than endothelial staining, these observations suggest that hypoglycosylated epitopes may also be regulated in other cell types that comprise lesions. Further studies are required to determine precisely the location and cell type dependence for changes in hypoglycosylated epitopes in atherosclerotic lesions.

The counter ligand on monocytes remains unclear, but numerous candidate mannose-recognizing proteins expressed on monocytes exist and include MBL, mannose receptor (MR), and dendritic cell–specific intercellular adhesion molecule-3-grabbing nonintegrin. MBL haplotype is associated with atherosclerotic risk levels, and MBL has been found in developing, but not advanced, murine and human lesions. Apart from binding mannose residues, MBL also functions in complement activation. Numerous members of the complement cascade and receptors are found in early atherosclerotic lesions, suggesting a role for complement in atherosclerotic development. Another mannose-recognizing protein found in atherosclerotic plaques is MR. MR is expressed on a variety of monocyte-derived cell populations, including alternatively activated M2 macrophages and dendritic cells. There is also evidence that MR is present on monocytes because ricin toxin–induced apoptosis of THP-1 monocytes can be attenuated with anti-MR antibodies. Lesion localized MR-expressing macrophages do not associate with lipids and do not transition into foam cells, suggesting that they are serving a nontraditional function within the plaques, possibly recruited to the region by the mannose residues on the luminal surface. Another family of proteins associated with atherosclerosis development are the c-type lectins known as the selectins (CD62e, CD62l, and CD62l). Selectins have established roles in monocyte rolling and adhesion and are known to bind complex N-glycans and O-glycans via sialyl Lewis x (sLe^x) motifs. However, evidence exists that they can interact with mannose, thus a role for the selectins as receptors of endothelial mannose cannot be ruled out.

Another protein expressed on monocytes, which is widely implicated in atherosclerosis and which can bind mannose, is the integrin heterodimer Mac1 (CD11b/CD18). Mac1 has been shown to bind type-I fimbriated Escherichia coli in a mannose-dependent manner, and CD11b+ monocytes show high binding affinity to mannosylated glycopolymers. Soluble CD11b demonstrates elevated binding to ICAM-1 produced in the presence of α-mannosidase inhibitors, suggesting that increased mannose on ICAM-1 promotes binding. Additional work has shown that anti-CD18 antibodies inhibit neutrophil binding to ECs cultured with α-mannosidase inhibitors. Collectively, this work establishes Mac1 as a potential MR on the surface of monocytes that could recognize hypoglycosylated N-glycans.

Although our data now show that endothelial hypoglycosylated N-glycans are involved in mediating monocyte adhesion, the precise glycoproteins carrying these carbohydrates are currently unknown. Previous studies provide some evidence as to which proteins may be involved. As mentioned, when produced in the presence of α-mannosidase type I inhibitors, ICAM-1 binds more effectively to Mac1. Interestingly, the 2 N-glycan sites flanking the Mac1-binding domain of ICAM-1 have the highest degree of N-glycan complexity of all ICAM-1 N-glycosylation sites, so addition of hypoglycosylated N-glycans at these sites could potentially increase binding. Another major regulator of monocyte adhesion is VCAM-1. Interestingly, different glycoforms of VCAM-1 are expressed in response to tumor cell conditioned media, suggesting that VCAM-1 might serve as a scaffold for proadhesive hypoglycosylated N-glycans. Supporting this concept, sialic acid, a sugar component of complex N-glycans, inhibits VCAM-1–dependent adhesion during flow. Finally, we note that effects of hypoglycosylated epitopes on increased monocyte adhesion were only revealed in the presence of flow, suggesting that adhesion molecule N-glycosylation differentially affects rolling versus firm adhesion. A traditional view is that rolling interactions are mediated by leukocyte selectins and endothelial P-selectin glycoprotein ligand 1, with ICAM-1 or VCAM-1 playing principal roles in firm adhesion. However, several studies using adhesion molecule–deficient mice or ECs derived from these have shown that ICAM-1 and VCAM-1 can also regulate monocyte rolling and firm adhesion especially at lower shear rates, which combined with data in Figure 3A, suggesting that ICAM-1 and VCAM-1 may be important targets for regulation by hypoglycosylation.

The fact that different stimuli induce different glycoforms of a protein is supportive of our previous work that demonstrated peroxisome proliferator–activated receptor-γ controlled N-glycosylation, independent of adhesion molecule expression. It is known that different glycoforms of a protein perform different functions in cells. P-selectin glycoprotein ligand 1 is known to bind P-selectin through an O-glycan linked α-2,3 sialic acid (ie, a glycan linked to a serine or threonine residue). Yet, despite this requirement for this binding, only
a small fraction of P-selectin glycoprotein ligand 1 contains sialic acid residues, implying that the majority of P-selectin glycoprotein ligand 1 does not serve as a selectin ligand. Similarly, specific functions of CD44, \(\gamma\)-aminobutyric acid, and IgG3 are determined by their glycosylation status, with different glycoforms of the proteins responsible for different functions. Thus, an interesting possibility arises where only a small pool of any given protein scaffold, or structurally similar proteins, contains the hypoglycosylated N-glycans that are regulating monocyte adhesion. Conversely, the addition of a specific glycan to a protein does not make it a ligand for a particular receptor. As mentioned, the selectins are known to bind to the sLe\(^x\) motif. However, the majority of proteins that contain sLe\(^x\) motifs are not ligands for selectin proteins, indicating that the saccharide structure itself is not sufficient to act as a ligand. Thus, a combination of the proper protein scaffold and specific carbohydrate(s) is critical for receptor recognition. This concept is highlighted in the current work as kif and swain increase mannose content (Figure 3E and 3F) but do not induce protein adhesion without further stimulation (Figure 4C and 4D), indicating that only mannose residues on certain proteins can behave as ligands for monocyte adhesion. In addition, the idea that different adhesion molecules undergo distinct N-glycosylation is demonstrated in Figure 3A. ICAM-1 undergoes distinct processing producing specific N-glycoforms in the presence of kif and swain. In contrast, VCAM-1 produces the same N-glycoforms when produced in the presence of kif and swain. These differences highlight how 2 major regulators of monocyte adhesion undergo distinct posttranslational processing.

In conclusion, evidence is provided that hypoglycosylated N-glycans are upregulated by proinflammatory oscillatory shear stress and are enriched in the vessel lumen during early atherosclerotic plaque development. Blocking mannose residues associated with these hypoglycosylated N-glycans with lectins attenuates, while increasing their expression with pharmacological inhibitors of N-glycan maturation, potentiates monocyte adhesion, thereby establishing endothelial mannos as a novel regulator of monocyte adhesion under flow.

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**Disclosures**

None.

**References**


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Role for endothelial N-glycan mannose residues in monocyte recruitment during atherogenesis

Running Title: Mannose regulates monocyte adhesion

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Cell Culture

HAECs were purchased from Lonza Corporation and cultured in EBM-2 + Bullet kit (Lonza, cc-3124) or MDCB-131 supplemented with 0.16-0.18 mg/ml bovine hypothalamus extract, 60 μg/ml heparin, 10% fetal bovine serum (FBS, HyClone) and penicillin(100U/ml)/streptomycin (100 μg/ml) and used between passages 3-6. All experiments were performed at one day post-confluence. THP-1 cells were purchased from ATCC and maintained in RPMI 1640 containing 10% FBS, 1mg/ml penicillin-streptomycin (all three from Invitrogen) at 0.5-1.0x106 cells/ml to maintain them in the log cell growth phase. For adhesion experiments either under static conditions or flow, monocytes were labeled with Cell-Tracker Green (Invitrogen) for 15min at 37°C.

Flow studies

Glass slides were coated overnight with bibronectin, blocked with 0.2% BSA, and cells were plated in flow media (MCDB-131 with 1% FBS). Slides were loaded onto a parallel plate flow chamber and subjected to different flow patterns in plating medium maintained at 37°C and perfused with 5% CO2. Laminar flow (12 dynes/cm²) was generated by gravity using a two reservoir system and a peristaltic pump as previously described. Oscillatory flow is generated using an infusion withdrawal pump (±5 dynes/cm², 1 Hz) with a 1 dyne/cm² forward flow superimposed by a peristaltic pump to facilitate nutrient/waste exchange.

Lectin staining

After treatments, cells were washed using ice cold PBS containing 1 mM each CaCl₂ and MgCl₂ and allowed to equilibrate for 5-10 min before staining. Cells were incubated for 10 min with 1 μg FITC-conjugated concanavalin a (ConA) or lens culinaris agglutinin (LCA) (Vector labs) and then washed three times with PBS. Fluorescence was measured using a Perkin Elmer Fluorescent plate reader. For determination of lectin-staining of cells exposed to shear stress, cells were stained as above and then fixed in 4% formaldehyde for 10 min and visualized on a Nikon Eclipse Ti inverted fluorescent microscope. Representative micrographs were collected using the Photometric CoolSNAP120 ES2 camera and fluorescence intensity was quantified using NIS Elements 3.00, SP5 imaging software.

Primary monocyte isolation
Mononuclear cells were isolated from fresh human blood collected with anticoagulant (Acid-Citrate-Dextrose, ACD) from healthy volunteers. Blood was layered over Histopaque- 1077 (Sigma Diagnostics) density gradient and centrifuged at 400 x g for 30 min at room temperature. The layer at the interface between plasma and the density gradient, which contains mononuclear cells and platelets was diluted 1:5 using PBS and centrifuged at 150 x g for 10 min to remove platelets. The pellet containing mononuclear cells was resuspended in RPMI (basal media). All protocols used for the collection and processing of blood samples were approved by the Institutional Review Board at University of Alabama at Birmingham.

**Static adhesion assay**

HAECs were grown in 48-well plates and treated as described for each experiment. Cells were then washed twice with warm sterile PBS and incubated with 60,000 Cell-Tracker Green labeled monocytes for 15 min at 37°C in a 5% CO2 incubator. Cells were gently washed three times with warm PBS and fluorescence was measured on a Victor² Perkin-Elmer Fluorescent plate reader (Exc=485nm, Em=535nm).

**Surface Elisa and cell staining**

HAECs were plated in black-walled 96-well plates or 12 mm glass cover slips in 24 well plates, allowed to reach confluency, and treated as described for each experiment. Cells were then fixed in 4% paraformaldehyde for 10 min and blocked in 5% goat serum in TBS for 30 min. Cells were incubated with mouse anti-ICAM1 (Abcam, ab2213) and anti-VCAM1 (Abcam, ab98954) at 1:250 in blocking buffer for 1 hr at room temperature. After washing, plates were incubated with goat anti-mouse Alexa488 (Invitrogen: A10667) at 1:500 in blocking buffer for 30 min at room temperature. Additionally, some cells were stained with secondary antibody alone to serve as background. After washing, fluorescence was detected on a Perkin-Elmer Fluorescent plate reader or viewed on a Leica DMRXA2 microscope equipped with a Hamamatsu C474-95-ERG camera.

**Flow Adhesion Assay**

Leukocyte rolling and firm adhesion during flow were determined using the Glycotech flow chamber system (Rockville, MD). HAECs were cultured in 35mm dishes and treated as described for each experiment. Plates were washed with warm sterile PBS and THP-1 or primary human monocytes (250,000 cells/ml) labeled with Cell Tracker Green were flowed over the endothelium at 100 µL/min corresponding to a wall shear rate of 0.5 dyne/cm² in RPMI basal
media (without serum) containing calcium and magnesium. The cells were viewed on a Leica inverted fluorescence microscope equipped with a Hamamatsu Orca ER digital CCD camera (Compix, Cranberry Township, PA). Real-time images of each field are captured at 20 frames/sec for one-three minutes. Resulting time-lapse images analyzed to calculate average rolling velocities. This was performed by motion tracking analysis using the Automated Image Capture and Motion Tracking and Analysis software (Simple PCI, Compix Inc, Cranberry Township, PA). Any cell that remained stationary ≥ 5-sec was considered to be firmly bound and numbers calculated per min of data acquired. Data are presented as fold change compared to mean TNFα for each experiment. Monocytes that had velocities below the critical rolling velocity (calculated as described previously\(^2\)) were considered as rolling cells and were measured over 1min.

**Enzymatic removal of carbohydrates**

For the selective removal of surface exposed high-mannose N-glycans cells were treated with TNFα (10 ng/ml, 4 hrs), washed with warm PBS, and then fixed in 4% paraformaldehyde for 10 min. Plates were washed and incubated with Endoglycosidase H (Glycozyme) (1 mU in 1 ml 100 mM Sodium Citrate pH 5.5) for 16 hr at 37°C.

**Lectin blocking experiments**

HAECs were treated with TNFα (10 ng/ml, 4 hrs) and then blocked with 2.5-10 µg lectin (LCA, ConA, or GNL) were added during the last 10 min of incubation. Monocyte adhesion under static and flow conditions were then determined as above.

**Immunohistochemistry**

Male ApoE -/- mice were purchased from Jackson Laboratories and fed standard chow diet for 8 weeks. At 16 weeks of age, mice were euthanized, perfused with 4% formaldehyde, and the innominate artery was excised. Human tissue was collected at the LSU-HSC during routine postmortem autopsy. All tissues were fixed in 4% formaldehyde, embedded in paraffin, and cut into 5 µm sections. Tissues sections were processed though xylenes (3x), 100% ethanol (2x), 95% ethanol, 85 % ethanol, 70% ethanol, and 50% ethanol for 5 min each. Slides were washed thoroughly with DI water and then placed in PBS containing 1 mM CaCl and 1 mM MgCl. Sections were incubated with 5 μg Rhodamine ConA or LCA for 30 min, washed 2 x in PBS, counterstained with Hoechst 33342, and washed 3 more times in PBS before mounting. For
blocking controls, 500 mM methyl-mannopyranoside was included for the duration of staining. Slides were viewed on a Leica DMRXA2 microscope equipped with a Hamamatsu C474-95-ERG camera using 10x and 40x objectives.

For PECAM-1 staining, sections were processed as above and antigens were retrieved in citrate buffer (10 mM tri-sodium citrate, 0.05% Tween 20, pH 6.0) for 45 min at 95°C. Sections were blocked with 10% goat serum in PBS for 30 min followed by incubation with rabbit anti-CD31 antibody (abcam-ab23684) 1:100 overnight in blocking buffer. Following washes, sections were incubated with goat anti-rabbit Alexa594 (Invitrogen, A110123) 1:500 for 30 min at room temperature. Sections were again washed, counterstained with Hoechst 33342, and mounted for viewing as above.

Western blot

After treatment, lysates were collected in 2x Lamelli buffer and boiled for 10 min. Protein samples were resolved on 4-12% PAGEr Gold Tris gels (Lonza) and transferred to nitrocellulose membranes (Bio-Rad). Blots were blocked with 2% heat denatured BSA in TBS + 0.1% Tween-20 (TBST) and incubated overnight at 4°C with primary antibodies in 0.5% heat denatured BSA (β-actin-1:10,000: Abcam-ab-6726, VCAM-1-1:2000: Santa Cruz-sc-8304, ICAM-1-1:2,000 Cell Signaling Technology, 4915). Blots were then washed 3 x with TBST, incubated with species appropriate HRP-conjugated secondary antibody (Santa Cruz) in the same buffer used for primary antibodies, washed 3 x with TBST, and bands were visualized using ECL substrate (Pierce) and x-ray film (Thermo Fisher).

Eastern blotting (lectin staining)

HAECs were kept static or exposed to oscillatory or laminar shear for 18 hours. Lysates were collected and resolved on SDS-PAGE gels and electroblotted onto nitrocellulose membranes. Membranes were incubated with 5 µg of FITC-ConA in PBS with 1mM CaCl and 1 mM MgCl for 1 hr at room temperature. Blots were extensively washed with PBS before imaging on a Typhoon TRIO variable mode imager using the 526nm-short-pass filter (GE Healthcare).

Statistical analysis

All experiments were conducted 3-6 times and significant differences were calculated as p<0.05 compared to control or TNFα by one-way ANOVA using GraphPad Prism 5.
Supplemental Figure I. Sections of the human carotid artery (A), innominate artery from 16wk ApoE mice on western diet for 8wk (B) or HAEC treated untreated (control) or treated with TNFα (10ng/ml, 4h) were stained with ConA or LCA in the presence or absence of 500 mM alpha-methylmannopyranoside (αMM). αMM inhibited ConA or LCA binding demonstrating mannose binding specificity for these lectins. Lectin staining was performed using rhodamine (red) conjugated ConA or LCA in Panels A and B, and FITC (green)-conjugated ConA or LCA in panels C.
Supplemental Figure II. HAECs were left static or exposed to laminar (LSS) or oscillatory shear (OSS) for 18 h. Lysates were prepared with SDS, resolved by gel electrophoresis and changes in ICAM-1 or VCAM-1 (Panel A) or high mannose epitopes determined by lectin blotting using FITC-ConA (Panel B). Shown are representative blots which indicate OSS-dependent increased expression of ICAM-1 and VCAM-1 and an overall increased binding of FITC-ConA to HAEC after exposure to OSS comprising of similar proteins with more ConA reactivity and new proteins (indicated by arrows).
Supplemental Figure III: HAECs were untreated or treated with 10 ng/ml TNFα for 4 hrs. Additionally, some cells were pretreated with kif (40 ng/ml) or swain (1 µM) alone for 2 hrs. The number of primary human monocytes rolling along the endothelium per minute was determined by measuring cells with rolling velocities below the critical rolling velocity as described in methods. Data show mean ± SEM (n=4) *P<0.05 relative to control or TNFα, **P<0.01 relative to control by 1-waw ANOVA with Newman Keuls post test.
**Supplemental Figure IV.** Human aortic endothelial cells were stimulated with TNFα (10 ng/ml, hrs) and monocyte adhesion was determined under flow. As indicated, cells were also incubated with 2.5-10 mg of ConA, GNL, or LCA for 5-10 minutes before monocyte adhesion was determined. *p<0.05 compared to TNFα by one-way ANOVA with Tukeys post test.
**Supplemental Table I**

**Human patient demographics**

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### Supplemental Table II

#### Human patient information

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References
