Identification of a high-mannose ICAM-1 glycoform: effects of ICAM-1 hypoglycosylation on monocyte adhesion and outside in signaling

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Scott DW, Dunn TS, Ballestas ME, Litovsky SH, Patel RP. Identification of a high-mannose ICAM-1 glycoform: effects of ICAM-1 hypoglycosylation on monocyte adhesion and outside in signaling. Am J Physiol Cell Physiol 305: C228–C237, 2013. First published May 22, 2013; doi:10.1152/ajpcell.00116.2013.—Endothelial adhesion molecules are critical effectors of inflammation ensuring coordinated interactions that allow leukocytes to home to sites of injury. These adhesion molecules are often extensively modified posttranslationally by the addition of N-glycans, but if, or how, these modifications contribute to the protein function remains poorly understood. Herein we show that activated endothelial cells express two distinct N-glycoforms of intercellular adhesion molecule 1 (ICAM-1) that comprise a complex N-glycoform with α,2,6 sialic acid present at relatively high levels and a second, less abundant and previously undescribed high-mannose glycoform (HM-ICAM-1). This novel HM-ICAM-1 glycoform was also detected in human coronary artery specimens and moreover appeared to be the dominant glycoform in vivo. Production of exclusively HM-ICAM-1 in cells by α-mannosidase inhibition increased monocyte rolling and adhesion compared with mature ICAM-1 consistent with high-mannose epitopes providing leukocyte ligands. Cross-linking of ICAM-1 transmits outside-in signals that affect endothelial permeability and survival. Interestingly, cell signaling (assessed using ERK, VE-cadherin, and Akt phosphorylation) was maintained after cross-linking of HM-ICAM-1 compared with mature ICAM-1; however, interactions with the actin cytoskeleton were lost with HM-ICAM-1. These findings suggest that specific ICAM-1 N-glycoforms modulate distinct aspects of the inflammatory response and identify HM-ICAM-1 as a new therapeutic target for controlling leukocyte trafficking and endothelial inflammation.

N-glycan; inflammation; adhesion molecule; endothelial cell

During inflammation, leukocytes must migrate across the vessel wall to reach underlying regions of disease (43, 52). This process involves rolling and adhesion steps that facilitate firm contacts between the circulating leukocytes and endothelial cells lining the vessel wall. The protein mediators of this process have been elucidated and based on these findings our current knowledge of the contributions of endothelial adhesion molecules such as ICAM-1, VCAM-1, and E-selectin have been defined (19, 32, 33). Interestingly, these adhesion molecules are all glycoproteins with extensive posttranslational modifications in the form of N-glycans.

Protein N-glycosylation is an enzyme-driven process that covalently attaches a core tetradesaccharide onto the amide residues of asparagines in an N-X-S/T sequon (X cannot be proline). Subsequent enzymes in the endoplasmic reticulum remove glucose and mannose before enzymes in the Golgi apparatus catalyze the addition of multiple monosaccharide subunits (fucose, galactose, N-acetylgalcosamine, and sialic acid) generating a panoply of N-glycan structures (45). N-glycans can be divided into three classes based on their monosaccharide content. These include high-mannose N-glycans, which contain five to nine mannose residues with no additional diversity (beyond the two core N-acetylgalcosamines); hybrid N-glycans, which contain three to five mannose residues and only one antennae of saccharides; or complex N-glycans with three mannose residues and two to four antennae of saccharides. As this process is constrained by linearity whereby mannose trimming must occur before addition of other monosaccharides, inhibition of mannose removal can result in increased production of high-mannose and hybrid N-glycan structures, which are considered to be hypo-N-glycosylated.

Under normal physiological conditions, the overwhelming majority of N-glycans are processed to the complex state (12); therefore, production of under processed N-glycans could be viewed as functionally impaired. Indeed, recent studies have reported that inflammation is associated with a decrease in N-glycan complexity and increased expression of high-mannose and hybrid structures (referred to as hypoglycosylated N-glycans; Refs. 21, 23, 31, 36). Moreover, genetic manipulation or pharmacological inhibition of N-glycan processing to limit N-glycan maturation modulates the inflammatory response (1, 4, 21) underscoring the importance of N-glycan branching in immune function. Recently, we demonstrated that proinflammatory stimulation of endothelial cells results in increased hypoglycosylated N-glycans on the cell surface (10, 41), possibly by inhibition of early mannose trimming (α-mannosidase) enzymes (34, 41). However, the proteins carrying these structures remain unknown.

Intercellular adhesion molecule 1 (ICAM-1, CD54) is a member of the Ig-like adhesion molecule superfamily and binds to the cognate leukocyte integrins LFA-1 (CD11a/CD18) and Mac1 (CD11b/CD18). ICAM-1 is known to mediate leukocyte rolling, adhesion, and transmigration (14, 17, 18, 32), and mice lacking functional ICAM-1 display decreased inflammatory disease progression (9, 11, 35). Moreover, ICAM-1 ligation and cross-linking elicit outside-in signaling that regulates endothelial permeability and survival (2, 15, 16, 20, 21, 29, 39, 48–50) highlighting the multiple functions of this protein in the immune response. Previous reports have demonstrated the ICAM-1 N-glycan content depends on the cell type in which the protein is produced (8) and specific ICAM-1 glycoforms are known to have altered function (13, 38). However, whether ICAM-1 N-glycosylation is controlled in endothelial cells during inflammation and whether this modulates...
the adhesive and signaling functions of ICAM-1 remain unclear.

Herein we identify a novel high-mannose ICAM-1 glycoform with distinct functions. We further demonstrate that HM-ICAM-1 is present in human arteries, show that it supports increased monocyte adhesion and maintains normal mitogenic cell signaling, but displays impaired interactions with the actin cytoskeleton.

**MATERIALS AND METHODS**

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Rockville, MD), THP-1 cells were purchased from ATCC (Rockville, MD), and Cos-1 cells were a generous gift of Joanne Murphy-Ulrich (University of Alabama, Birmingham). MCDB-131, DMEM (low glucose), HI-FBS, trypsin-EDTA, and L-glutamine were purchased from Lonza (Rockville, MD), THP-1 cells were purchased from ATCC (Rockville, MD), and Cos-1 cells were maintained in DMEM containing 10% FBS, penicillin (100 U/ml)/streptomycin (100 μg/ml) or heparin, and penicillin (100 U/ml)/streptomycin (100 μg/ml) and used between passages 3–7. Cos-1 cells were maintained in DMEM containing 10% FBS and penicillin (100 U/ml)/streptomycin (100 μg/ml). THP-1 cells were maintained in RPMI 1640 containing 10% FBS, penicillin (100 U/ml)/streptomycin (100 μg/ml), and 2 mM L-glutamine.

**Cell culture.** HUVECs were propagated on gelatin-coated dishes in MCDB-131 containing 10% FBS, 25 μg/ml endothelial cell growth supplement (BD Biosciences), 1 mM L-glutamine, 30 μg/ml heparin, and penicillin (100 U/ml)/streptomycin (100 μg/ml) and used between passages 3–7. Cos-1 cells were maintained in DMEM containing 10% FBS and penicillin (100 U/ml)/streptomycin (100 μg/ml). THP-1 cells were maintained in RPMI 1640 containing 10% FBS, penicillin (100 U/ml)/streptomycin (100 μg/ml), and 2 mM L-glutamine.

**Generation of pCMV-ICAM-1 and stable transfections.** pQCXIN-FLAG-ICAM-1 was obtained from Laurent Coscoy (University of California, Berkeley) and subcloned into pCMV-Script using the NotI and EcoRI sites. Lipofectamine 2000 (Invitrogen) was used for transfections according to the manufacturer’s protocols. Cos-1 cells were selected in media supplemented with 750 μg/ml G418 for 3 wk.

**Glycosidase digestion.** Endothelial cells were treated as described and digested with endoglycosidase H (Endo H) or peptide-N-glycosidase F (PNGaseF; both from Prozyme, Hayward, CA) according to the manufacturer’s protocol.

**Coronary artery protein isolation.** Coronary arteries were obtained post mortem, the adventitia was carefully and thoroughly removed, and the vessel (~2 cm segment) was lysed (50 mM Tris pH 2.4, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM MgCl₂, 1 mM CaCl₂, and 1 mM PMSF) using 50 strokes of a dounce homogenizer before being cleared by centrifugation at 14,000 g for 10 min. The supernatant was collected and incubated with concanavalin A (Con A; high-mannose binding lectin) agarose (with a small fraction reserved for loading control) for 4 h with gentle rocking before being washed extensively with lysis buffer. Bound proteins were released by boiling in SDS-PAGE sample buffer before being resolved for Western blot analysis.

**Table 1. Biotinylated lectins used and their glycan binding specificities**

<table>
<thead>
<tr>
<th>Lectin Name</th>
<th>Acronym</th>
<th>Sugar Specificity</th>
<th>Glycan Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concanavalin A</td>
<td>ConA</td>
<td>Man⁠₁–⁠₆(Man⁠₁–⁠₃)Man</td>
<td>Hybrid N-glycan</td>
</tr>
<tr>
<td>Lens culinaris agglutinin</td>
<td>LCA</td>
<td>Fuc⁠ α₁–⁠₄GlcNAc, α₁–⁠D-Glc, α₁–⁠D-Man</td>
<td>Hybrid N-glycan</td>
</tr>
<tr>
<td>Sambucus nigra lectin</td>
<td>SNA</td>
<td>Sia0,2-6GalNAc</td>
<td>Complex O-glycan</td>
</tr>
<tr>
<td>Maackia amurensis lectin</td>
<td>MAA</td>
<td>Galβ4GlcNAcβ6 (GlcNAcβ2Man3/Man3)</td>
<td>Complex N-glycan</td>
</tr>
<tr>
<td>Phaseolus vulgaris agglutinin</td>
<td>PHA-L</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE.

**Adhesion assay.** Adhesion assays were performed as previously described (41). For static assays, HUVECs or Cos-1 cells were grown in 48-well plates and treated as described for each experiment. Cells were washed with warm PBS and incubated with 6 × 10⁶ CellTracker Green labeled THP-1 monocytes for 30 min at 37°C. Plates were gently washed with PBS and fluorescence was measured on a Victor® Perkin-Elmer Fluorescent plate reader (Exc = 485 nm and Em = 520 nm).

![Figure 1](http://ajpcell.physiology.org/) Identification of a concanavalin A (ConA)-reactive intercellular adhesion molecule 1 (ICAM-1) glycoform on activated endothelial cells. A: human umbilical vein endothelial cells (HUVECs) were stimulated with TNF-α (10 ng/ml, 6 h) with or without pretreatment with swainsonine (swain) or kifunsine (kif). Lysates were analyzed by Western blot analysis for ICAM-1 and β-actin expression. B: HUVECs were stimulated with TNF-α as above and labeled with biotinylated lectins (ConA, SNA, MAA, PHA-L, or LCA; see Table 1 for definitions) and purified against streptavidin. Whole cell lysates (WCL) and streptavidin bound surface proteins were analyzed by Western blot analysis for ICAM-1 and β-actin expression. Data are representative of at least 4 separate experiments.
Leica DMi600B fluorescent microscope equipped with a Hamamatsu OrcaER digital camera.

Membrane fractionation. HUVECs grown on 35-mm culture dishes were treated as described for experiments and lysed in 100 μl TST (25 mM Tris-pH 7.4, 150 mM NaCl, 0.02% Triton X-100, and protease inhibitor cocktail) for 5 min on ice. Lysates were cleared at 14,000 g for 3 min, and the supernatant was kept as the soluble fraction. The pellet was washed in TST and cleared at 14,000 g for 3 min. The washed pellet was then lysed in 100 μl of TST containing 60 μM β-octylglucopyranoside for 20 min at 37°C and collected at 20,000 g for 5 min. The resulting supernatant was considered the Triton X-100 insoluble fraction. Equal volumes from each fraction were analyzed by Western blot analysis.

Immunoprecipitation. For interaction with the Ezrin-Radixin-Moe- sin (ERM) complex, ICAM-1 immunoprecipitations were performed as previously described (25). Briefly, proteins were collected in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, and protease inhibitor cocktail), cleared by centrifugation at 14,000 g for 5 min, and incubated with Protein A Dynal beads (Invitrogen) and 1 μg mouse anti-ICAM-1 (Abcam-ab2213) overnight at 4°C with gentle rocking. Beads were washed three times with lysis buffer, and bound proteins were released by boiling in SDS-PAGE sample buffer. A small portion (~10%) of the original lysate was reserved for input control.

For interaction with caveolin-1, ICAM-1 immunoprecipitations were performed as previously described (25). Briefly, cells were treated and lysed in modified RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 0.5% NP-40, 1 mM MgCl₂, 1 mM sodium orthovanadate, and protease inhibitor cocktail) and further processed as above.

Western blotting. Samples were resolved on 4–15% TGX gels (Bio-Rad, Hercules, CA) and transferred to PVDF membranes. Blots were blocked with 5% milk in TBS + 0.1% Tween-20 (TBST) and incubated overnight at 4°C with antibodies against ICAM-1 (Abcam-15067), caveolin-1 (ab22130), caveolin-2 (ab18766), and moesin (ab54000). Blots were incubated with HRP-conjugated secondary antibodies (1:2000 in PBS) for 1 h, washed, and detected with ECL luminescent substrate (GE Healthcare). Membranes were imaged with a ChemiDoc XRS+ device (Bio-Rad) and analyzed by Image Lab software (Bio-Rad). Protein expression levels were quantified using a standard curve generated using known amounts of purified protein.
Identification of two ICAM-1 glycoforms in activated endothelium. TNF-α stimulation of HUVECs produces two distinct ICAM-1 bands; a major band at ~95 kDa and a minor and lower molecular mass band at ~75 kDa (Fig. 1A). The lower band migrates at a similar size to ICAM-1 produced in the presence of the class 1 α-mannosidase inhibitor kifunensine, suggesting that this is a hypoglycosylated ICAM-1 bearing exclusively high-mannose N-glycan structures. To determine the N-glycan structures of ICAM-1 on the surface of activated endothelial cells, HUVECs were stimulated with TNF-α (10 ng/ml, 6 h) and surface labeled with a panel of biotinylated lectins with distinct carbohydrate specificities (Table 1). Immunoprecipitation using streptavidin was then performed followed by blotting for ICAM-1. In parallel whole cell lysates were subject to the same procedure. As seen in Fig. 1B, labeling with the mannose specific lectin ConA selectively pulled down the lower molecular mass glycoform of ICAM-1 from the surface. Enrichment of this ICAM-1 glycoform was not observed with any other lectin tested. In contrast, the higher molecular mass ICAM-1 glycoform interacts with SNA but not ConA, LCA, PHA-L, or MAA. This lectin binding pattern suggests the absence of α-2,3 sialic acid or fully branched structures but supports the presence of α-2,6 sialic acid structures on the higher molecular mass ICAM-1, consistent with previous reports (22). Selectivity of lectins was confirmed by performing pull-downs in cells treated with the α-mannosidase class I inhibitor kifunensine, which produces only high-mannose structures that only reacted with ConA, and with the α-mannosidase class II inhibitor swainsonine, which produces hybrid N-glycan structures that reacted with LCA and SNA but not with ConA or PHA-L.

Lower molecular mass isoform of ICAM-1 contains high-mannose N-glycans. To confirm that the lower molecular mass isoform of ICAM-1 contains high-mannose structures, HUVECs were stimulated with TNF-α as before and lysates were collected and digested with glycosidases. Specifically, lysates were digested with the pan-N-glycosidase PNGaseF or the high-mannose-specific Endo H. As seen in Fig. 2, Endo H treatment selectively removed the lower molecular mass glycoform converting it into a ~53-KDa species, which equates to the predicted molecular mass of ICAM-1 based on its primary amino acid sequence. Endo H had no effect on the higher molecular mass ICAM-1, confirming the assignment that the ~75-KDa form is a high-mannose ICAM-1 (HM-ICAM-1) N-glycoform. Enzyme specificity was verified by the ability of Endo H to digest all of ICAM-1 produced in the presence of kifunensine and to partially digest ICAM-1 produced in the presence of swainsonine. Note that ICAM-1 produced in the presence of swainsonine and digested by Endo H appeared as multiple bands by Western blot analysis highlighting the heterogeneity of glycan structures added to the protein even at the hybrid stage.

**HM-ICAM-1 is present in vivo.** To assess if HM-ICAM-1 was present in vivo and not an artifact of tissue culture, freshly isolated human coronary arteries were lysed, and ConA reactive proteins isolated and processed as described in MATERIALS AND METHODS. As seen in Fig. 3A, the protein profile of ConA bound proteins was strikingly different compared with that from the whole cell lysate indicating 1) that ConA enriched proteins, and 2) the presence of a number of high-mannose N-glycan containing proteins. We next performed Western blot analysis on these samples and, as seen in Fig. 3B, were...
able to detect HM-ICAM-1 in the ConA pull-down samples. The molecular mass of this band was ~75 kDa, which corresponds to the ConA-reactive band from TNF-α-stimulated endothelial cells and indicates that HM-ICAM-1 is present in vivo.

**HM-ICAM-1 supports monocyte adhesion with increased adhesion under flow.** We next determined if HM-ICAM-1 could support monocyte adhesion. For these experiments, HUVECs were transfected with either empty vector (pCMV-script) or pCMV-ICAM-1 to overexpress ICAM-1. Some cells were also treated with kifunensine starting at the time of transfection to generate exclusively HM-ICAM-1. Forty-eight hours after transfection, HUVECs were incubated with THP-1 monocytes and adhesion assessed. As seen in Fig. 4A, kifunensine treatment alone had no effect on monocyte adhesion. However, expression of ICAM-1 in these cells significantly increased THP-1 adhesion, with similar levels seen in the presence or absence of kifunensine but that did not reach the level of adhesion seen with TNF-α treatment (data not shown). Because primary endothelial cells are difficult to transfect (~50% efficiency), which resulted in lower levels of ICAM-1 expression compared with endogenous induction with TNF-α, these experiments were repeated in stably transfected Cos1 cells. As seen in Fig. 4B, kifunensine treatment alone did not increase THP-1 adhesion to Cos1 cells but overexpression of ICAM-1 or HM-ICAM-1 lead to equally robust monocyte adhesion. Collectively these data demonstrate that HM-ICAM-1 possesses normal function in regards to static monocyte adhesion.

Previously, we demonstrated that high-mannose epitopes on the endothelial surface potentiated TNF-α-induced monocyte adhesion under conditions of flow (41). We next examined THP-1 monocyte adhesion under flow to ICAM-1-expressing Cos1 cells in the presence or absence of kifunensine. As seen in Fig. 4C, addition of kifunensine significantly increased THP-1 monocyte adhesion in Cos1 cells transfected with pCMV-ICAM-1. There was no detectable adhesion in empty

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**Fig. 5.** HM-ICAM-1 supports cell signaling. HUVECs were treated with kifunensine for 0–32 h, and the effect on VE-cadherin and PECAM-1 N-glycosylation was determined by Western blot analysis (A). HUVECs were treated with TNF-α in the presence of absence of kifunensine (B) and cells then treated with THP-1 monocyte for 15 min and VE-cadherin phosphorylation was detected by Western blot analysis. Shown are representative blots and bar graphs showing means ± SE (n = 3). *P < 0.01, relative to respective control by one-way ANOVA with Tukey posttest. C and D show respectively phosphorylation of Akt and ERK after cross-linking of ICAM-1 as described in MATERIALS AND METHODS. Shown are representative blots with bar graphs showing means ± SE (n = 3). *P < 0.01, relative to respective control by one-way ANOVA with Tukey posttest.
vector transfected cells in the presence or absence of kifunensine (data not shown).

**HM-ICAM-1 supports cell signaling.** Antibody-mediated clustering or leukocyte engagement to ICAM-1 is known to induce a wide range of cell signaling events as outlined above. To determine if HM-ICAM-1 could support similar signaling we first examined THP-1 monocyte induced VE-cadherin phosphorylation, which was previously shown to require endothelial expression of ICAM-1 (2). However, it was critical to assess these functions before N-glycosylation inhibitors had affected the glycosylation status of constitutively expressed proteins. HUVECs were treated with kifunensine for 0–32 h, and the expression of constitutively expressed VE-cadherin and PECAM-1 was assessed by Western blot analysis. As seen in Fig. 5A, high-mannose glycoforms of these two proteins only become evident between 8–12 h after treatment, and complete expression of the high-mannose forms was not seen until at least 24 h. For this reason, all experiments were conducted before 8 h of kifunensine exposure to only affect newly synthesized proteins. As seen in Fig. 5B, incubation of TNF-α-stimulated endothelial cells, in the presence or absence of kifunensine, with THP-1 monocytes led to an increase in VE-cadherin tyrosine phosphorylation. To further determine if HM-ICAM-1 could support cell signaling we examined Akt and ERK phosphorylation after antibody-mediated clustering as previously described (28, 29). As seen in Fig. 5, C and D, cross-linking of HM-ICAM-1 induced Akt and ERK phosphorylation comparable to fully glycosylated ICAM-1. Combined, these results indicate that HM-ICAM-1 can support cell signaling equally compared with native ICAM-1.

**HM-ICAM-1 displays reduced interaction with cytoskeletal components.** Previous reports indicate that ICAM-1 can interact with the actin cytoskeleton through interactions with the ERM complex (28) or through interactions with filamin A upon clustering which forms a complex with caveolin-1 (32). We next determined if HM-ICAM-1 could interact with moesin or caveolin-1 as previously described. Figure 6A shows that moesin was coimmunoprecipitated from TNF-α-stimulated

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**Fig. 6.** HM-ICAM-1 displays impaired interactions with the actin cytoskeleton. HUVECs were treated with TNF-α (10 ng/ml, 6 h) in the presence of absence of α-mannosidase inhibitors swainsonine or kifunensine. A: ICAM-1 was immunoprecipitated as described in experimental procedures for interaction with the Ezrin-Radizin-Moesin (ERM) complex. Whole cell lysates (input) and immunoprecipitated proteins were analyzed by Western blot analysis. B: cells were treated as before and some cells underwent antibody-mediated ICAM-1 clustering. ICAM-1 was immunoprecipitated as described in MATERIALS AND METHODS for interaction with caveolin-1. Whole cell lysates (input) and immunoprecipitated proteins were analyzed by Western blot analysis. C: cells were treated and some cells underwent antibody-mediated ICAM-1 clustering (X-ICAM). Lysates were separated into Triton X-100 soluble (S) and insoluble (I) fraction and analyzed by Western blot. Data are representative of at least 3 different experiments. D: cells were treated and underwent antibody-mediated clustering of ICAM-1. After fixation, cells were stained with fluorescently tagged secondary antibody and surface expressed patterns of ICAM-1 were assessed. Data are representative of at least 3 different experiments.
HUVECs but was not present in samples pretreated with swainsonine or kifunensine. Furthermore, ICAM-1 clustering induced coimmunoprecipitation of caveolin-1 with ICAM-1 but was markedly reduced in cells pretreated with kifunensine (Fig. 6B).

Previous reports have also shown that upon antibody-mediated clustering of cell surface ICAM-1, the latter transitions from Triton X-100 soluble to insoluble membrane fractions (33, 34). Based on our finding that HM-ICAM-1 could not interact with the ERM complex, nor with caveolin-1 when clustered by antibodies, we hypothesized that it would fail to transition to Triton X-100 insoluble membranes after antibody-mediated clustering. Figure 6C shows that both fully processed ICAM-1 and HM-ICAM-1 are found in Triton X-100 soluble fractions before clustering. After clustering, the fully processed ICAM-1 completely enters the Triton X-100 insoluble membrane fraction whereas only a portion of HM-ICAM-1 is able to make this transition. Collectively, these data suggest that despite reaching the membrane, HM-ICAM-1 displays impaired interactions with the actin cytoskeleton and lateral movement on the cell surface. This is further indicated by representative immunofluorescence images of ICAM-1 distribution on the cell surface (Fig. 6D), which show that a punctate focal distribution for mature ICAM-1 but a more diffuse pattern for ICAM-1 produced in the presence of swainsonine or kifunensine.

**DISCUSSION**

In the current work we have shown that ICAM-1 exists as two N-glycans on the surface of activated endothelial cells; a fully processed form containing terminal α-2,6 sialic acid and a minimally processed or hypoglycosylated high-mannose form (Figs. 1 and 2). Further, we have demonstrated that HM-ICAM-1 is detectable in human coronary arteries (Fig. 3), whereas only a portion of HM-ICAM-1 is able to make this transition. Collectively, these data suggest that despite reaching the membrane, HM-ICAM-1 displays impaired interactions with the actin cytoskeleton and lateral movement on the cell surface. This is further indicated by representative immunofluorescence images of ICAM-1 distribution on the cell surface (Fig. 6D), which show that a punctate focal distribution for mature ICAM-1 but a more diffuse pattern for ICAM-1 produced in the presence of swainsonine or kifunensine.

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To date several studies have examined the N-glycan structures on ICAM-1, but these studies have relied on plasmid-driven overexpression of the protein rather than examination of endogenous production (8) or examined murine but not human ICAM-1(37). In the latter case, mouse ICAM-1 was overexpressed in three cell lines where the protein plays no relevant function in leukocyte trafficking. Thus, while these data provide a great deal of information, they do not provide insight into ICAM-1 function. Underscoring this perspective is that glycan modifications of a single protein is dependent on the host cell type that in turn imparts unique functions to that protein. As examples, the HIV protein gp120 overexpressed in a panel of immortalized cells retains the N-glycan signature of the host cell type and this host cell glycan signature regulates envelope recognition (40). ICAM-2 produced in platelets contains terminal α-2,3 sialic acid that prevents DC-SIGN and LFA-1 binding (51). Interestingly, ICAM-2 produced in endothelial cells does not contain α-2,3 sialic acid. With respect to ICAM-1, previous studies have shown that murine astrocyte production of macrophage inflammatory protein-2 could be stimulated by murine ICAM-1 when produced in CHO cells, but not when produced in HEK cells, a property dependent on the N-glycan signature (38). The latter study also demonstrated that a high-mannose ICAM-1 was unable to stimulate astrocytes but was able to bind LFA-1 at normal levels. Elegant work by laboratory of Springer (13) has demonstrated that deletion of two consensus N-glycan sequences that flank the Mac-1 binding domain in the IgG like-3 domain of ICAM-1 (N240 and N269) enhanced Mac-1 binding. Thus glycosylation of ICAM-1 is a posttranslational modification that can regulate interactions with other proteins.

However, while these studies have clearly shown that N-glycosylation can control ICAM-1 function, it is important to note that the N-glycosylation patterns of human and murine ICAM-1 are drastically different. Human ICAM-1 contains 9 while murine ICAM-1 contains 10 N-X-S/T motifs, yet only 4 of the motifs are conserved between the species (42). Of note, the two glycosylation sites in ICAM-1 that are known to regulate Mac1 binding (13) are absent in murine ICAM-1. Additionally, a single mutation at position 20 of human ICAM-1 deletes an N-glycosylation site in the IgG like-1 domain that allows rhinovirus binding (7). Thus extrapolating information from murine ICAM-1 N-glycosylation patterns and applying it to human ICAM-1 is difficult. Many studies that have examined leukocyte binding to ICAM-1 have utilized recombinant protein produced in a variety of cells types but rarely from human (or primate) cells. In many cases, these
experiments use recombinant human ICAM-1 produced in murine myeloma cells, which we have observed does not contain HM-ICAM-1 (not shown). Apart from the absence of HM-ICAM-1, these proteins also contain the N-glycan signature of the host cell line (murine myeloma) (8) and may not reflect the action of ICAM-1 produced in an activated human endothelial cell. Figure 3 demonstrates that HM-ICAM-1 can be detected in human coronary arteries, and unlike in cultured endothelial cells where two ICAM-1 glycoforms were present (refer to Figs. 1 and 2), only one glycoform, the HM-ICAM-1 glycoform, was detectable in these samples, even in the whole tissue homogenate. A limitation in this measurement is that we cannot discern whether the HM-ICAM-1 detected is endothelial specific or derived from another cell type. That said, this observation is exciting in that it suggests that the majority of ICAM-1 in vivo is of the high-mannose variety and thus represents the actual therapeutic target rather than the often studied complex (higher molecular mass) N-glycan containing glycoform. This possibility remains to be tested.

Importantly, HM-ICAM-1 was equally effective as fully matured ICAM-1 to engaging monocytes under static conditions. During flow, however, HM-ICAM-1 was significantly more potent at mediating monocyte adhesion. Future experiments will be required to determine if these mannose residues are ligands for receptors other than Mac-1 and LFA-1 or if other mannose binding receptors involved in flow-mediated adhesion, such as DC-SIGN, are participating in monocyte adhesion to HM-ICAM-1. Taken together, we speculate that HM-ICAM-1 is the primary mediator of ICAM-1-dependent monocyte rolling and adhesion in vivo and consistent with our previous studies showing that endothelial surface HM-epitopes provide ligands for monocytes (10, 41).

Beyond monocyte adhesion, our data also suggest that the N-glycan complement of ICAM-1 regulates outside in signals. Whereas ICAM-1 ligation-dependent effects on mitogenic signals (indexed by AKT or ERK phosphorylation) or junctional integrity (indexed by VE-cadherin phosphorylation) were not different between HM-ICAM-1 or fully mature ICAM-1, basal interactions with the actin cytoskeleton via ERM complexes and lateral movement of ICAM-1 on the cell-surface were dramatically different and lost with HM-ICAM-1. While these results may seem contradictory, that signaling is maintained while lateral mobility is lost, it is important to note that outside-in signaling after ICAM-1 cross-linking is abolished by cytochalasin D while lateral mobility is not (39, 46). In this sense, outside-in signaling requires new stress fiber formation whereas lateral mobility requires preexisting interactions with the cytoskeleton (such as those mediated by interactions with the ERM complex; illustrated in Fig. 7). Data in Fig. 6C demonstrate that HM-ICAM-1 has impaired ability to transition to Triton X-100 insoluble membranes after cross-linking but also show that some HM-ICAM-1 is already in these fractions before cross-linking. This is in direct contrast to mature ICAM-1, which is only found in Triton X-100 soluble membranes before cross-linking. Thus it would appear that surface distribution, at least in the context of membrane domains, is altered in HM-ICAM-1, a conclusion further indicated by Fig. 6D. The functional effects of these differential interactions with cytoskeletal components require further testing as do studies to define which of the eight human N-glycosylation sites are required for these functions. Of particular interest are sites at N358 and N379, which lie in the region where ICAM-1 dimerization is reported to occur (53). N-glycosylation is known to regulate dimer formation of many proteins (6, 24, 26, 27, 44), so it is easy to speculate that ICAM-1 N-glycosylation could be important in this context and could thereby regulate leukocyte adhesion and clustering events.

To conclude, the current work demonstrates that ICAM-1 exists in two distinct N-glycoforms on activated endothelial cells in vitro with a major form carrying α-2,6-sialic acid possessing complex N-glycans and a minor form carrying only high-mannose N-glycans. This high-mannose glycoform is abundantly present, however, in human coronary artery samples and when overexpressed in cells is able to support monocyte rolling and adhesion. However, the high-mannose form fails to interact with the ERM complex and displays reduced lateral motility upon clustering despite the ability to support outside-in signaling (Fig. 7). Collectively, these data identify a HM-ICAM-1 as a novel ICAM-1 glycoform that mediates critical functions of this protein and targeting of which offers potentially novel therapeutic strategies to control endothelial inflammation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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