Minimally modified low-density lipoprotein induces monocyte adhesion to endothelial connecting segment-1 by activating β1 integrin

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We have shown previously that treatment of human aortic endothelial cells (HAECs) with minimally modified low-density lipoprotein (MM-LDL) induces monocyte but not neutrophil binding. This monocyte binding was not mediated by endothelial E-selectin, P-selectin, vascular cell adhesion molecule-I, or intercellular adhesion molecule-I, suggesting an alternative monocyte-specific adhesion molecule. We now show that moncytic α4β1 integrins mediate binding to MM-LDL-treated endothelial cells. We present data suggesting that the expression of the connecting segment-1 (CS-1) domain of fibronectin (FN) is induced on the apical surface of HAEC by MM-LDL and is the endothelial α4β1 ligand in MM-LDL-treated cells. Although the levels of CS-1 mRNA and protein were not increased, we show that MM-LDL treatment causes deposition of FN on the apical surface by activation of β1integrins, particularly those associated with α5 integrins. Activation of β1 by antibody 8A2 also induced CS-1-mediated monocyte binding. Confocal microscopy demonstrated the activated β1 and CS-1 colocalize in concentrated filamentous patches on the apical surface of HAEC. Both anti-CS-1 and an antibody to activated β1 showed increased staining on the luminal endothelium of human coronary lesions with active monocyte entry. These results suggest the importance of these integrin ligand interactions in human atherosclerosis.


Introduction

There is considerable evidence that oxidized lipoproteins play a role in the development of atherosclerosis. Early studies showed oxidized lipoproteins to be present in atherosclerotic lesions (1–3). Studies on hyperlipidemic animals demonstrated that oxidation products were increased in lesions (4). In addition, antioxidants such as probucol and butylated hydroxytoluene have been found to reduce the development and severity of lesions in a number of animal models (5, 6).

Mononuclear cell adhesion to vascular endothelium was observed in the initial steps of fatty streak formation (7, 8). Recent studies (9, 10) using mice null for monocyte chemotactic protein-1 (MCP-1) and the MCP-1 receptor have demonstrated the important role of monocytes in lesion development. Our group has used minimally modified low-density lipoprotein (MM-LDL)–stimulated aortic endothelial cells to model the development of the fatty streak (11). We have demonstrated that monocyte, but not neutrophil, adhesion and transmigration across the aortic endothelium is increased in response to MM-LDL (11). Active oxidized phospholipids isolated from MM-LDL were found to be increased in rabbit atherosclerotic lesions (12).

Previous studies from our group have identified MM-LDL–induced molecules involved in several phases of monocyte/endothelial interactions. Previous work (13, 14) suggests that in vivo entry of leukocytes into the vessel wall involves at least three steps: rolling, activation, and firm adhesion to the endothelium. The rolling step has been shown to involve the interaction of selectins on the endothelium, with their ligands on leukocytes. Studies from our group and others (15–18) suggest that P-selectin is an important rolling molecule for monocytes in atherosclerosis. Using in vitro studies, we have shown that levels of P-selectin in human aortic endothelial cells (HAEC) are increased by MM-LDL (18), whereas levels of E-selectin are decreased (19). We and others have also shown that highly oxidized low-density lipoprotein (LDL) leads to P-selectin release to the upper cell surface (18, 20). Specific cytokines and chemokines that activate monocyte adhesion ligands have been found in lesions.
onto nitrocellulose. Membranes were probed with polyclonal FN (lane 614). In a chymotryptic digest of pFN (5 μg/lane), the 90.45 antibody recognized a band at ~66 kDa (lane 4) with an intensity greater than its recognition of intact pFN (compare lanes 3 and 4) (a). To compare the pattern of antibodies, whole cell lysates prepared in RIPA lysis buffer were separated and transferred onto nitrocellulose. The polyclonal FN (lane 5), monoclonal FN (lane 6), 90.45 (lane 7), and 7E5 antibodies (lane 8) detected two bands of 220 and 190 kDa. For the monoclonal and polyclonal FN antibodies, the 220-kDa band stained with a greater intensity (lanes 5 and 6) than the 190-kDa band, whereas the CS-1 and 7E5 antibodies recognized the 190-kDa band better than the 220-kDa band (lanes 7 and 8) (b). FN, fibronectin; RIPA, radioimmunoprecipitation assay.

Methods
Characterization of the reactivities of the FN antibodies used for these studies. Because the studies presented here use FN and CS-1 antibodies for different experimental procedures, these antibodies were compared to determine the characteristics of each antibody. Four different antibodies against FN were used for these studies: (a) IgM monoclonal antibody against the CS-1 region of FN (CS-1 90.45, referred to as 90.45; Cytel Corp., San Diego, California, USA; refs. 47, 48); (b) 7E5, a second CS-1 IgM monoclonal antibody that has the same reactivity to peptides and FN as the previously reported CS-1 polyclonal antibody (Ugarova, T., personal communication; ref. 49); (c) monoclonal FN antibody (specific for a cell-binding region on the eighth type III repeat; catalog no. MAB1937; Chemicon International, Temecula, California, USA; ref. 50); and (d) polyclonal rabbit anti–human FN (IgG) antibody (catalog no. 341640; Calbiochem-Novabiochem Corp., San Diego, California, USA). Western blotting was performed testing the recognition of plasma (catalog no. FC010; Chemicon International) and endothelial cell FNs by these antibodies. For these studies, endothelial cells were solubilized with a combination of detergents and protease inhibitors (25 mM Tris-HCl [pH 8.0], 0.15 M NaCl, 1% Triton X-100, 0.5% NaDOC, 5 mM EDTA, 0.1% SDS, 1 mM PMSF, 10 μg/ml aprotinin, 1.5 μg/ml pepstatin A, and 10 μg/ml leupeptin) for 1 h at 4°C. Extracts were diluted into loading buffer (50 mM Tris-HCl [pH 6.8], 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) and boiled for 5 min before being separated on a 7.5% SDS-PAGE; then the proteins were transferred onto nitrocellulose membranes.
were incubated with the antibodies at a concentration of 1–2 
\( \mu \)g/ml. Lower antibody concentrations and shorter exposure 
times were required for the polyclonal FN and monoclonal 
antibodies to the cell-binding region of FN (1 h), compared 
with those required for the monoconal antibodies to CS-1 FN 
(overnight). Blots were developed using secondary antibodies 
conjugated to horseradish peroxidase (HRP) for viewing with 
chemiluminescence (ECL; Amersham Life Sciences Inc., 
Arlington Heights, Illinois, USA).

**Determination of CS-1 FN mRNA levels.** A lysate RNase assay kit 
(United States Biochemical, Cleveland, Ohio, USA) was used to 
determine the message levels of HAEC either unstimulated or 
stimulated with 250 \( \mu \)g/ml MM-LDL for 4 h at 37°C. Cells were 
centrifuged, the supernatant was removed, and the cell pellets 
were dissolved in guanidine thiocyanate lysis solution (106 
mM guanidine, 5 mM mercaptoethanol, 0.5% sodium deoxycholate, 
4%–6% polyacrylamide/7 M urea gel). Once the bromophenol 
blue dye marker had migrated to the bottom of the gel, it was 
removed and exposed to Kodak film (Eastman Kodak Co., 
Rochester, New York, USA) for 24 h.

**Testing of agents that block monocyte/endothelial cell interactions in vitro.** The basic methods for isolation and culture of HAEC and 
preparation of MM-LDL have been described previously (37).

For all of the following experiments, HAEC grown in a 48-well 
dish (catalog no. 3548; Corning-Costar Corp., Acton, Massa-

chussets, USA) were either untreated or treated for 4 h at 37°C with 
MM-LDL (250 \( \mu \)g/ml) in 5% FBS (catalog no. A-1111-1; 
HyClone Laboratories, Logan, Utah, USA) in 5% Medium 199 
catalog no. 1902, Irvine Scientific, Santa Ana, California, USA).

Human peripheral blood monocytes were isolated, and adhe-
sion was tested as described previously (51). Three types of inhibi-
tion experiments were performed: (a) treatment of monocytes 
with antibody or peptide; (b) treatment of HAEC with MM-
LDL or 8A2 \( \beta 1 \)-activating antibody followed by treatment with 
blocking antibodies; and (c) treatment of HAEC with blocking 
antibodies before the addition of MM-LDL. For all inhibition 
experiments, a range of antibody concentrations were tested 
(data not shown), with 5 \( \mu \)g/ml yielding maximum inhibition. 
For the first type of blocking experiments, monocytes were pre-
treated for 30 min at room temperature with antibodies to 
either \( \alpha 4 \) (L25, catalog no. 550019; Becton Dickinson Immunocy-
tomery Systems, San Jose, California, USA), \( \beta 1 \) (PSD2; gift 
of E. Wayner, University of Minnesota Medical School, 
Minneapolis, Minnesota, USA; ref. 52), \( \beta 2 \) (TS/18; American Type 
Culture Collection, Rockville, Maryland, USA), or \( \beta 7 \) (gift of A. 
Lazarovits, University of Western Ontario, Ontario, Canada; ref. 
53); or with either 500 \( \mu \)g/ml CS-1 active (peptidomimetic of 
the CS-1 region of FN) or 500 \( \mu \)g/ml scrambled control peptide 
(Cytel Corp.; ref. 48) before addition to the endothelial cells for 
15 min. In the second type of experiments, HAEC were incu-
bated for 4 h as untreated control, or separately, with 250 \( \mu \)g/ml 
MM-LDL and 3 \( \mu \)g/ml of 8A2, or with 2 \( \mu \)g/ml of LPS as a 
positive control. After the incubation, the endothelial cells were 
treated for 30 min with a 5 \( \mu \)g/ml blocking concentration of 
either VCAM-1 (BBA-S; BRI, Oxford, United Kingdom), 90.45, 
7E5, or polyclonal FN antibody before the addition of mono-
cytes. A monocyte binding assay was performed, and unar-

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**Figure 2**

Agents that block monocyte/endothelial interactions *in vitro*. HAEC were 
stimulated with 250 \( \mu \)g/ml MM-LDL at 37°C for 4 h. Human monocytes 
were incubated with 5 \( \mu \)g/ml anti-\( \alpha 4 \), \( \beta 1 \), \( \beta 2 \), or \( \beta 7 \) for 30 min before 
addition to the endothelial cell layer. Unbound monocytes were rinsed 
off and the cells fixed with 1% glutaraldehyde in 1X PBS. Anti-\( \alpha 4 \) and anti-
\( \beta 1 \) both significantly reduced the number of monocytes bound to 
the endothelial cell layer. Neither anti-\( \beta 2 \) nor anti-\( \beta 7 \) demonstrated 
an effect on the level of monocytes bound (*a*). In a separate study, HAEC were 
treated as just described and monocytes were incubated with either active 
CS-1 (500 \( \mu \)g) or scrambled peptide (500 \( \mu \)g) for 20 min at room tem-
perature. A binding assay was performed as just described. The active CS-
1 peptide significantly reduced the levels of bound monocytes, whereas 
the scrambled peptide did not affect the levels bound (*b*). In the last 
experiment, HAEC were treated with MM-LDL, as described, before being 
incubated with blocking antibodies to VCAM-1 (4B9), polyclonal FN, 
90.45, or 7E5 at 5 \( \mu \)g/ml for 30 min. Untreated monocytes were used to 
perform the binding assay. The antibody against VCAM-1 did not reduce 
the number of monocytes bound to MM-LDL–stimulated HAEC. How-
ever, FN, 90.45, and 7E5 antibodies all significantly reduce monocyte 
adhesion (*c*). For each condition, the number of monocytes was deter-
mined by visually counting four fields per well in four separate wells. Addi-
tionally, all experiments are representative of four separate studies. Val-
ues represent mean ± SD (n = 12). *P < 0.0005. CS-1, connecting 
segment-1; HAEC, human aortic endothelial cells; MM-LDL, minimally 
modified low-density lipoprotein; VCAM-1, vascular cell adhesion mole-
cule-1; APBS, PBS + BSA; GNS, goat normal serum.
were plated into 96 wells and then stimulated with 250 μg/ml of MM-LDL or 2 ng/ml LPS for 4 h at 37°C. After the treatment, cells were rinsed and then fixed with 4% paraformaldehyde for 20 min at room temperature. Cells were rinsed and then blocked for 1 h with 3% APBS. Primary antibodies were diluted to 1 μg/ml in the blocking solution and then incubated overnight at 4°C. Cells were then rinsed and blocked with GNS before incubation with either the IgM (90.45) or IgG (VCAM-1) secondary antibodies conjugated to HRP. Development was with o-phenylene-diamine dihydrochloride in a phosphate citrate buffer. The plate was read on a microtiter plate reader at an OD of 450 nm. A representative experiment from four separate studies is shown. Values represent mean ± SD (n = 4). *P < 0.0001. HRP, horseradish peroxidase.

Figure 3
MM-LDL stimulates surface expression of CS-1 but not VCAM-1. HAEC were incubated for 4 h at 37°C without or with MM-LDL or 2 ng/ml LPS for 4 h at 37°C. After the treatment, cells were rinsed and then fixed with 4% paraformaldehyde for 20 min at room temperature. Cells were rinsed and then blocked for 1 h with 3% APBS. Primary antibodies were diluted to 1 μg/ml in the blocking solution and then incubated overnight at 4°C. Cells were then rinsed and blocked with GNS before incubation with either the IgM (90.45) or IgG (VCAM-1) secondary antibodies conjugated to HRP. Development was with o-phenylene-diamine dihydrochloride in a phosphate citrate buffer. The plate was read on a microtiter plate reader at an OD of 450 nm. A representative experiment from four separate studies is shown. Values represent mean ± SD (n = 4). *P < 0.0001. HRP, horseradish peroxidase.

ELISA: Cells in 96-well dishes (catalog no. 3595; Corning Costar Corp.) were incubated for 4 h at 37°C without or with MM-LDL diluted to 250 μg/ml, 2 ng/ml LPS, 3 μg/ml 8A2 (β1-activating antibody; provided by N.L. Kovach, University of Washington, Seattle, Washington, USA; ref. 54), or 1.5–3 mM MnCl₂ for the times indicated in each experiment. For some experiments, cells were treated with 200 ng/ml of cycloheximide (CHX) for 30 min before the treatments listed earlier here. After incubation of HAEC with various activators, the wells were rinsed with 1× PBS containing Ca/Mg before being fixed in 4% paraformaldehyde for 20 min at room temperature. After the fixative, the cells were thoroughly washed to remove excess antibody before adding untreated monocytes to the wells.

For each inhibition study, four separate experiments were performed. In each experiment, four fields from each of four wells were visually counted for adherent monocytes under an inverted microscope with an eyepiece grid.

Immunofluorescence confocal microscopy: For these studies, glass coverslips (catalog no. 12-545-100; Fisher Scientific Co., Pittsburgh, Pennsylvania, USA) placed into a 12-well dish (catalog no. 3512; Corning-Costar Corp.) were used. The procedure of coating the coverslips with Cell Tak (catalog no. 40240; Becton Dickinson, Bedford, Massachusetts, USA) and Vitrogen 100 (catalog no. 0701-1N; Collagen Aesthetics, Palo Alto, California, USA) promoted HAEC adhesion without changing the characteristics of the cells. Cell Tak was mixed 1:1 with 2 M Na₂CO₃ and 5 μl was placed into the center of each coverslip. The Cell Tak/Na₂CO₃ mixture was then spread evenly over the entire surface of the coverslip by using a sterile plastic cell scraper. Vitrogen (1 ml/well of a 12-well dish diluted 1:50 into sterile 1× PBS) was then added to the wells, and coverslips were incubated overnight at 37°C before plating cells. For immunofluorescence, cells were fixed with 4% paraformaldehyde and processed as described earlier. For permeabilization of the membrane, some cells were incubated with 0.5% Triton X-100 for 5 min at room temperature. The 90.45, 7E5, HUTS-21, and monoclonal FN primary antibodies were used. For single immunofluorescence using 7E5, cells were incubated overnight at 4°C. The antibody was then viewed using a secondary antibody conjugated to CY-3 (red). For double immunofluorescence between 90.45 and the monoclonal FN antibodies, cells were first incubated overnight at 4°C with 90.45 antibody, and the next day, with the monoclonal FN antibody for 2 h at room temperature. Antibody was removed and cells rinsed before adding secondary antibodies for both 90.45 [αlgM F(ab')2; CY3; red] and monoclonal FN antibody [αlgG F(ab')2; FITC; green] (catalog no. 115-095-006; Lampire Biological Pipersville, Pennsylvania, USA) for 1 h at room temperature. For double immunofluorescence between 90.45 and HUTS-21, the cells were incubated with the 90.45 antibody overnight at 4°C. The next day, the 90.45 antibody was rinsed off and the cells were incubated with HUTS-21 for 2 h at room temperature. The 90.45 was recognized first using αlgM FITC (green)–conjugated secondary antibody (catalog no. F0205; DAKO Corp.). Afterward, the HUTS-21 was detected using CY3 (red)–conjugated IgG secondary antibody (catalog no. 115-166-003; Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA). Control experiments using the secondary antibodies in the reverse order did not affect the overall results (data not shown). For double immunofluorescence between CS-1 recognized by the 90.45 antibody and wheat germ agglutinin (WGA), a well-characterized membrane marker that recognizes N-glycosaminoglycans, cells were incubated with 90.45 and IgM CY3 secondary antibody as already described here. The cells were rinsed and then incubated for 2 h at room temperature with WGA conjugated to FITC (catalog no. FL-1021; Vector Laboratories, Burlingame, California, USA). After rinsing and mounting with Vectashield immunofluorescence solution (catalog no. H1000; Vector Laboratories), the edges of Raleigh, North Carolina, USA) in 3% APBS. Other cells were incubated for 1 h with monoclonal FN antibody (0.5 μg/ml) or with HUTS-21 (to detect activated β1; provided by C. Cahill at ref. 55) for 2 h (2 μg/ml). In a separate ELISA, the following primary antibodies were used at 1 μg/ml for 2 h: anti-α3, anti-α5, anti-αv, anti-β1, and anti-β3. After the incubation with primary antibody, the wells were rinsed and blocked thoroughly before they were exposed to a HRP-conjugated secondary antibody. After the incubation, cells were rinsed again with 1× PBS followed by double distilled H₂O. The peroxidase color reaction was developed in the dark using OPD (catalog no. P8787; Sigma Chemical Co., St. Louis, Missouri, USA). The plate was then read on a Molecular Devices (Menlo Park, California, USA) kinetic microtiter plate reader using a Softmax 881 L-1 end-point program (Molecular Devices) at an OD of 450 nm. The antibody concentration and incubation times were optimized to ensure testing in the linear range.
the coverslip were sealed using clear nail polish. Confocal microscopy was performed on the single- and double-labeled immunofluorescent samples to determine the conformation and relative locations of the 7ES, WGA, 90.45 with argon ion (488 nm excitation) and helium neon green lasers (543 nm excitation) and it was used to make optical sections of the cell samples. A Z series made up of several consecutive 0.5-μm-thick sections were taken starting at the apical side and ending at the basal side for analysis of the preparation.

Immunohistochemistry on human endothelial lesions. Frozen sections of human aortic endothelium were stained for the presence of 90.45, HUTS-21, VCAM-1, CD 31 (catalog no. M0823; DAKO Corp.) or HAM 56 (catalog no. EAB-935; ENZO New York, New York, USA) in lesions. The tissues were fixed with acetone for 5 min at 4°C, and slides were blocked for 1 h at room temperature with serum of the same species as the secondary antibody. Sections were then incubated for various times with the primary antibody (diluted to 1–2 μg/ml). After the antibody incubation (see Fig. 10 a–d), the sections were rinsed and stained with biotinylated secondary antibody. Endogenous peroxidase activity was blocked with a 20-min incubation of 0.3% H2O2/MeOH solution. Antibodies were viewed using ABC (catalog no. PK6100; Vector Laboratories) and AEC (catalog no. SO1; BiomedicaFoster City, California, USA) kits. For Figure 10, e and f, dianaminobenzidine was used to view the antibodies.

**Figure 4**
Confocal series for WGA, 90.45, 7ES, and monoclonal FN antibodies on nonpermeabilized cells. HAEC were treated with MM-LDL for 4 h at 37°C before being fixed and processed as nonpermeabilized cells. Optical sections were taken starting at the apical surface where 90.45 staining was most prominent. Cells were double labeled with 90.45 viewed by CY3 (red) and with the membrane marker WGA conjugated to FITC (green). The levels of both 90.45 and WGA were most intense on the apical side (red) and with the membrane marker WGA conjugated to FITC (green). Staining was most prominent. Cells were double labeled with 90.45 viewed by CY3 (red) and with the membrane marker WGA conjugated to FITC (green). The levels of both 90.45 and WGA were most intense on the apical side (red) and with the membrane marker WGA conjugated to FITC (green). Staining was most prominent. Cells were double labeled with 90.45 viewed by CY3 (red) and with the membrane marker WGA conjugated to FITC (green). The levels of both 90.45 and WGA were most intense on the apical side (red) and with the membrane marker WGA conjugated to FITC (green). Staining was most prominent.

**Results**
Characterization of the FN antibodies used for these studies. A previous study (49) has shown that a polyclonal antibody to the CS-1 peptide reacted more strongly with fragmented than intact plasma FN. We used Western blotting to characterize the reactivity of the CS-1 monoclonals used in this study with plasma and cellular FN (Fig. 1). The polyclonal FN (lane 1), monoclonal FN antibody (lane 2), and CS-1 90.45 (lane 3) all detected a single band at ~220 kDa in plasma FN (5 μg/lane) (Fig. 1a). As expected, the FN monoclonal antibody displayed much stronger recognition of intact plasma FN (5 μg/lane). A chymotryptic digest of FN (5 μg) showed that the CS-1 90.45 antibody recognized a band at ~66 kDa (lane 4) and that the intensity of the band was much higher when compared with intact plasma FN (Fig. 1a, compare lanes 3 and 4). The antibody recognition of cellular FN was then examined using lysates of endothelial cultures (Fig. 1b). FN polyclonal antibody (lane 5), FN monoclonal antibody (lane 6), CS-1 monoclonal antibody 90.45 (lane 7), and CS-1 monoclonal antibody (lane 8) all recognized two bands from whole cell lysates, one at ~190 kDa. Others have shown that FN antibodies can recognize molecules of 220 kDa and 190 kDa (49, 56), the latter being formed by fragmentation with certain proteases. As expected, the CS-1 monoclonals reacted more strongly with the neoepitope present in the 190-kDa band, which is likely exposed by fragmentation of the 220-kDa FN. The FN antibodies reacted more strongly with the larger molecule. These results in HAEC contrast with observations in astrocytes, where the 90.45 antibody recognized a 300-kDa band not

**Figure 5**
Pretreatment of HAEC with antibodies against α5 and β1, but not α3, αv, or β3, inhibited MM-LDL-mediated monocyte binding. HAEC were pretreated for 30 min with 5 μg/ml of blocking antibodies to α5, α3, αv, β1, and β3 before addition of MM-LDL for 4 h at 37°C (in the continuing presence of antibody). The cells were rinsed extensively, and a monocyte binding assay was performed. The MM-LDL-induced increase in monocyte binding was significantly blocked back to control levels by antibodies to α5 and β1 but not α3, αv, or β3. The data presented are representative of four experiments. Values represent mean ± SD (n = 12). *P < 0.001.

**Statistical analysis.** For all experiments described here, data were analyzed using the Statview 4.5 program (Abacus Concepts Inc., Berkeley, California, USA). All P values were calculated using ANOVA and Fisher’s protected least significant difference test.

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MM-LDL increased the activation of β1 without increasing the total amounts of α5β1 on the apical surface. HAEC were either untreated or treated with MM-LDL for 4 h. ELISA was performed on nonpermeabilized cells to detect the apical expression of α5, α3, β3, and β1. MM-LDL did not increase the total levels of integrins on the surface of the HAEC (a). Values represent mean ± SD (n=4). Using HUTS-21 to detect the activated form of β1 integrin, ELISA demonstrated that MM-LDL increased the amount of β1 activation in a time-dependent fashion (b). Values represent mean ± SD (n=4). *P < 0.0001. Each experiment is representative of four separate studies.

**Figure 6**

**MM-LDL increases**

The amount of active form of the present mean Values represent mean a5b1 amounts of b1 MM-LDL increased the activation of Figure 6

- **Effect of integrin antibodies, CS-1 FN antibodies, and active CS-1 peptide on MM-LDL–induced monocyte binding.**

  To determine the monocye integrins responsible for the adhesion to MM-LDL–treated HAEC were performed by incubating monocytes, before their addition to the HAEC, with either control antibody or with antibodies against α4, β1, β2, and β7 integrin subunits. Control antibody was not effective at reducing levels of MM-LDL–induced monocyte binding (Fig. 2a). However, the level of monocyte binding to MM-LDL–treated cells was reduced by incubation of monocytes with antibodies against α4 and β1 but not with antibodies against β2 and β7 (P < 0.0005) (Fig. 2a). Further confirmation for a role for VLA-4 was obtained by exposing monocytes to a peptidomimetic of the CS-1 region of FN, a known inhibitor of VLA-4 interaction with its ligands (58). The active peptide reduced the number of adherent monocytes by ~76% (after subtraction of control), whereas the scrambled control peptide did not reduce the number bound (P < 0.0005) (Fig. 2b). These results indicated that the VLA-4 integrin present on the surface of monocytes was involved in MM-LDL–induced monocyte adhesion to the endothelial cell surface.

  To identify the endothelial ligand for monocyte VLA-4, the MM-LDL–treated endothelial cells were exposed to antibodies against VCAM-1 and FN, the two known alternative ligands for VLA-4. Endothelial cells were treated for 4 h with MM-LDL and then exposed for 30 minutes to antibody. The antibodies were washed off, and the monocytes were added to the treated HAEC. A monoclonal blocking antibody against VCAM-1 (4B9) significantly blocked lipopolysaccharide (LPS)–induced (P < 0.0005; data not shown), but not MM-LDL–induced, monocyte binding (Fig. 2c). To determine the role of CS-1 in MM-LDL–induced binding, MM-LDL–treated HAEC were exposed to two different monoclonal antibodies against the 25-amino acid CS-1 sequence, as well as a polyclonal antibody to FN (Fig. 2c). MM-LDL–stimulated endothelial cells that were treated with either the 90.45 or 7E5 antibody before the addition of monocytes showed a significant reduction (70%–75% reduction) in the level of monocyte binding (P < 0.0005), whereas irrelevant antibody did not reduce levels of binding. In addition, a polyclonal antibody to FN was also effective at reducing levels of MM-LDL–induced monocyte binding (61% reduction). The antibodies did not have an effect on monocyte binding to untreated cells (data not shown). These data strongly suggest that MM-LDL induces monocyte binding by the interaction of monocyte VLA-4 with CS-1 FN on the surface of endothelial cells.

**MM-LDL stimulates CS-1 apical surface expression in HAEC.** We next examined the ability of MM-LDL to increase CS-1 at the level of mRNA, protein and cell-surface expression. RNase protection assay did not detect an increase in the levels of CS-1 message after MM-LDL treatment (CO = 1.76, MM = 1.70; data expressed as CS-1/GAPDH ratio). In addition, Western blotting with radioimmunoprecipitation assay (RIPA) lysis buffer demonstrated that levels of CS-1 FN total protein (matrix plus cell-associated) were not increased in MM-LDL–treated cells (CO = 0.4, MM = 0.3; data expressed as CS-1/tubulin ratio). However, treatment of endothelial cells with MM-LDL, but not LPS, significantly increased apical surface expression of CS-1 by enzyme-linked immunosorbent assay (ELISA) on nonpermeabilized attached cells using the 90.45 antibody (Fig. 3). (The confocal studies described later here showed that antibody bound only to the apical surface.) Levels of surface expression were increased ~1.7–3-fold over a series of several experiments. Similar results were seen with 7E5 (data not shown). Levels of VCAM-1 were not increased by MM-LDL on these HAEC (Fig. 3). Previous studies by our group (37) demonstrated the ability of CHX to reduce monocyte adhesion to MM-LDL–stimulated cells. Cell-surface ELISA demonstrated that the addition of CHX significantly reduced the increase in CS-1 surface expression after MM-LDL stimulation (60% reduction, P = 0.0175; data not shown). These series of experiments demonstrates that MM-LDL increases the
expression of FN on the apical surface without increasing the levels of total FN message or protein and that this process depends on protein synthesis.

Characterization of CS-1 localization by immunofluorescence confocal microscopy. Immunofluorescence confocal microscopy was used to determine the location of the FN recognized by the 90.45, 7E5, and monoclonal FN antibodies. Evidence for CS-1 localization to the apical surface was obtained by double immunofluorescence using the 90.45 antibody and WGA, a well-characterized membrane marker. Fluorescein isothiocyanate (FITC)–labeled WGA was found to colocalize with CS-1 on the apical surface by confocal microscopy (Fig. 4a). The distribution of FN localized by the 90.45 and monoclonal FN antibodies in MM-LDL–treated cells was examined under higher magnification (Fig. 4b–d). Thin optical sections were made starting at the apical surface where CS-1 staining was most prominent. To provide better orientation, the cell is outlined in white on the figure. At the apical surface, the 90.45 antibody stained a patchlike configuration located adjacent to the nucleus (Fig. 4b, inset). This type of staining pattern was also observed using 7E5 (Fig. 4b). The monoclonal FN antibody also stained the filamentous patch on the apical surface (Fig. 4c) in addition to binding to fibrillar strands on other parts of the apical surface and on the cell periphery (data not shown). There was clear colocalization of the monoclonal FN antibody within the CS-1 patch, which is seen by yellow color of the combined fluorochromes (Fig. 4d). The colocalization was not due to an artifact of double immunofluorescence, because the fluorochromes used have emissions that are sufficiently separated from one another to avoid overlap in detection (FN; FITC 492 nm and CS-1; CY3 550 nm). Colocalization, therefore, can be attributed to the recognition of the same molecule by both antibodies. Permeabilization of cells with Triton X-100 further confirmed that patches were localized to the apical surface. In permeabilized cells, the 90.45 antibody demonstrated cytoplasmic staining of intracellular FN (data not shown). The patterns of antibody staining described here were observed in cells cultured in serum containing FN, as well as in FN-free serum (data not shown).

Treatment of HAEc with anti-α5 and anti-β1, but not anti-α3, anti-αv, or anti-β3, before incubation with MM-LDL, inhibited monocyte binding and CS-1 expression. We next examined the ability of MM-LDL to increase the retention of CS-1 FN on the apical surface by activation of FN-binding integrins. HAEc were pretreated with blocking antibodies to integrins previously shown to mediate FN binding to the cell surface: VLA-5, α3β1, and αvβ3. HAEc were pretreated for 30 minutes with 5 μg/ml of blocking anti-integrin antibodies to α5, α3, αv, β1, and β3 integrins and then incubated with antibody and MM-LDL for four hours. The cells were washed thoroughly to remove the antibodies, and then a monocyte binding assay was performed. The MM-LDL–induced increase in monocyte binding was significantly inhibited (back to control levels) by antibodies to α5 and β1, but not by anti-α3, αv, or β3 integrins (Fig. 5). In fact, both αv and β3 antibodies actually stimulated binding to MM-LDL–treated cells. Treatment of control cells with the anti-α5 or anti-β1 antibodies did not decrease monocyte binding (data not shown). Addition of anti-α5 to the cells 90 minutes after MM-LDL addition was ineffective at inhibiting binding (data not shown). Treatment of HAEc with anti-α5 and anti-β1 antibodies before MM-LDL stimulation also significantly reduced the surface expression of CS-1 as determined by ELISA on nonpermeabilized HAEc (MM-LDL = 82.9 ± 6.78; α5 + MM = 21 ± 6.21, and β1 + MM = 18.5 ± 4.80, expressed as percent increase in OD above untreated cells ± SD). These findings suggest that functional α5 and β1 integrins comprising VLA-5 are important for the MM-LDL–induced CS-1 expression on HAEc.

MM-LDL increased activation of β1 integrins but not the total amount of α5β1 on the apical surface. The induction of CS-1 expression by MM-LDL could be mediated by either increased expression or activation of VLA-5 (28). An ELISA performed using antibodies that have equal affinity for both the activated and the nonactivated state of the integrins detected no increase in the levels of α5, β1, α3, or β3 in response to MM-LDL (Fig. 6a). Several antibodies have been described recently (55) that specifically detect the activated conformation of β1 integrins. Another ELISA was performed using the HUTS-21 antibody (55) to detect apical surface expression of the activated β1 epitope. A time-dependent increase in the expression of the activated β1 epitope on the MM-LDL–treated cells was observed (Fig. 6b). The maximum
expression (three- to fourfold increase) of this epitope was seen at four hours and remained elevated for at least six hours (data not shown). Cells treated with MnCl₂ for 15 minutes were used as a positive control because MnCl₂ has been shown to activate β₁ integrins rapidly (Fig. 6b). This time course of expression of activated β₁ is similar to that observed for monocyte binding as published previously (11). An assay using a different β₁ activation detection antibody, 15/7 (59), gave similar results (data not shown). Confocal immunofluorescent studies were performed using HUTS-21 antibody to detect the distribution of activated β₁ integrin on the apical surface. In control cells, there was a low level of staining where a few clusters of activated β₁ were observed (Fig. 7a). Cells treated with MM-LDL (Fig. 7b) demonstrated a marked increase in the number of β₁ clusters with usually only one cluster per cell. This image is a visual representation of the significant increase in β₁ activation quantitated by ELISA (Fig. 6b). The cells treated with MnCl₂ showed a diffuse increase in staining of activated β₁ integrins with fewer and smaller clusters (Fig. 7c). Currently, there is no antibody available to detect the activated form of αβ. However, because we were able to inhibit MM-LDL-induced monocyte adhesion by pretreatment with antibodies against αβ and β₁ and not other anti-integrin antibodies, it is likely that the activated β₁ that mediates FN binding in response to MM-LDL is associated with αβ.

Treatment of endothelial cells with an antibody that activates β₁ integrins increased monocyte binding and CS-1 expression. To determine whether an alternative method of β₁ activation could also lead to increased CS-1 expression and monocyte binding, an antibody that activates β₁ integrins (8A2) was used (54). HAEC were treated with 3 μg/ml of 8A2 antibody for periods of 15 minutes to four hours. After treatment, the cells were thoroughly rinsed to remove the antibody, and a monocyte binding assay was performed using human monocytes. HAEC treatment with 8A2 induced monocyte binding in a time-dependent manner, with a maximum increase (about three- to fourfold) seen at the end of four hours (Fig. 8a). That monocyte binding was not increased after treatment for 15 and 30 minutes indicates that rinses to remove the activating antibody from HAEC were sufficient to avoid direct activation of monocytes by 8A2. In a separate set of experiments, 8A2 did not induce neutrophil binding (data not shown). The 8A2-induced monocyte binding at four hours was completely inhibited by treatment of monocytes with anti-αβ antibody but not by a nonspecific IgG (8A2 = 122 ± 7.1; 8A2 + αβ = 34 ± 6.0; 8A2 + IgG = 128 ± 6.5 monocytes per field). An ELISA on nonpermeabilized cells demonstrated that at four hours of treatment, 8A2 did not significantly increase VCAM-1 expression, whereas there was a significant increase in the apical surface expression of CS-1 (Fig. 8b). To confirm further a role for CS-1 in 8A2-mediated monocyte binding, the effect of the 90.45 antibody was examined. Cells were treated for four hours with 8A2, exposed to the 90.45 antibody, and rinsed, and monocyte binding was measured. The 90.45 antibody inhibited monocyte binding of monocytes to 8A2 treated by 55% but caused no significant reduction in monocyte binding to untreated cells (Fig. 8c). From these studies, we conclude that, like MM-LDL, activation of β₁ integrin by antibody increases monocyte binding by inducing retention of CS-1 FN on the apical cell surface.

CS-1 and activated β₁ integrin colocalize on the apical surface of HAEC. Confocal microscopy on double immunofluorescence was performed on HAEC stained for both CS-1...
(monoclonal antibody IgM 90.45, FITC green–conjugated secondary) and activated β1 (monoclonal antibody IgG HUTS-21, CY3 red–conjugated secondary). Both CS-1 (Fig. 9a) and β1 (Fig. 9b) were found on the upper cell surface in a patchlike and cluster configuration, as seen in Figs. 4b and 7b. CS-1 and activated β1 were found to colocalize together on the cell surface and appear as yellow (Fig. 9c). Patches were most frequently found on the apical surface near the nuclear periphery (Fig. 9d), although some patches overlapped the nuclear surface.

**Immunohistochemistry of human coronary lesions.** Studies were performed on 15 different coronary lesions taken from five different individuals to compare 90.45, VCAM-1, and HUTS-21 staining on luminal endothelium. All sections examined stained positively for CD 31, which detected the presence of endothelium (Fig. 10a); some regions also stained positively for HAM 56, which detected the presence of macrophages (Fig. 10b). In 13 of 15 vessels, areas where staining was positive for both CD 31 and HAM 56, staining for 90.45 was observed on the luminal endothelium (Fig. 10c). In areas lacking HAM 56, the luminal endothelium on only two vessels was positively stained. VCAM-1 staining was not seen on the luminal endothelium in 13 of 15 vessel areas with HAM 56–positive staining (Fig. 10d) but was seen in one area that stained negative for HAM 56. Sections incubated with the 90.45 antibody preabsorbed to the active CS-1 peptidomimetic did not yield any observable staining (data not shown). A separate study was performed on seven different coronary vessels, one each from four different individuals and three from a fifth individual, to determine whether similar areas demonstrated staining of luminal endothelium for HUTS-21 (Fig. 10f) and 90.45 (Fig. 10g). HUTS-21 staining was generally more extensive than 90.45, going slightly deeper into the intima. However, in six of the seven vessels, areas of highest intensity of 90.45 and HUTS-21 were very similar but not completely identical (Fig. 10e and f, arrow and double arrow). We noted that the endothelial cells in areas that were positive for HUTS-21 and 90.45 were taller and exhibited more nuclei per square centimeter, which suggested activation. However, it was not possible to determine by immunohistochemistry whether both the CS-1 and HUTS-21 staining were localized to the apical surface of the endothelium. These studies do suggest that staining of luminal endothelium in areas where monocyte/macrophages are present is higher for 90.45 than VCAM-1 and that most 90.45 staining occurs in areas of staining for activated β1 integrin.

**Discussion**

The goal of our studies was to determine the ligands on the monocyte and endothelium responsible for enhanced binding to endothelial cells treated with MM-LDL. Monocytes express a variety of surface integrins that they use to bind to the endothelial cell layer (60). Using blocking antibodies, we demonstrated that VLA-4 was the major monocyte integrin responsible for MM-LDL–induced binding (Fig. 2a). We present several lines of evidence that MM-LDL induces monocyte binding to endothelial CS-1 FN, one of the two major ligands previously identified for VLA-4. Treatment of endothelial cells with MM-LDL induced the apical expression of CS-1 FN (Fig. 3). Exposure of monocytes to an active but not scrambled CS-1 peptide strongly inhibited binding to MM-LDL–treated endothelial cells (Fig. 2b). However, this result is not definitive because VCAM-1 binding is also blocked by the CS-1 peptide, although to a lesser extent (48). More importantly, treatment of endothelial cells with two different antibodies to the CS-1 region of FN and with a polyclonal antibody to FN strongly inhibited the binding of monocytes to MM-LDL–treated cells (Fig. 2c). In the present studies, immunofluorescence on nonpermeabilized HAEC demonstrated that different CS-1 monoclonal antibodies recognized filamentous patches on the upper cell surface located adjacent to the nucleus (Fig. 4b, inset). These patches were also recognized by a monoclonal FN antibody (Fig. 4c). Additionally, these patches correspond to regions where most monocytes have been found to bind in MM-LDL–treated cells (data not shown). It has been demonstrated that binding of VLA-4 to CS-1, like the binding of VCAM-1 to VLA-4, represents firm adhesion and requires that VLA-4 be either fully or partially activated (61). Studies by Weber and colleagues (62) demonstrated that CS-1 is able to function as a firm adhesion molecule under flow conditions. Furthermore, the adhesive strength of VLA-4 on isolated human monocytes for a CS-1 containing
FN fragment was rapidly increased and then reduced by MCP-1 (62). Previous studies by our group (24–26) have demonstrated that MCP-1 and other monocyte activators are increased by MM-LDL treatment of endothelial cells. We hypothesize that although a low level of CS-1 is expressed by untreated HAEC, it is not involved in monocyte binding to these cells owing to lack of monocyte VLA-4 activators. Thus our data suggest that the firm adhesion molecule induced by MM-LDL on endothelial cells is CS-1 FN; this molecule causes binding to the activated monocyte VLA-4 ligand.

The mechanism by which MM-LDL induces apical expression of FN was examined in a series of experiments. MM-LDL did not increase alternative splicing of FN, nor did it increase levels of total FN (cell-associated plus matrix-associated) as seen by RNase protection assay and Western blots of RIPA lysates. Rather, we provide extensive evidence that MM-LDL increases retention of FN on the apical surface of the cell. Peters and colleagues (63) have shown that endothelial cells secrete FN constitutively along the apical and the basal surface in culture. The process of FN binding to cells has been extensively studied in fibroblasts (64), and several cell-surface integrins have been shown to mediate FN assembly (45, 64–67). Our results demonstrate that treatment of endothelial cells with blocking antibodies to endothelial integrins α5 and β1, but not α3, αv, or β3, before stimulation with MM-LDL, blocked the induction of monocyte binding and CS-1 expression (Fig. 5; see Results) indicating that VLA-5 mediated FN binding. Binding of FN to VLA-5 has been shown to occur via the RGD-containing cell-binding domain of FN (68). Our results in endothelial cells are different from an earlier report (69), which noted that native LDL and very-low-density lipoprotein (VLDL) increased FN binding to MG-63 osteosarcoma cells by threefold via exposure of the FN–FN association sites. MM-LDL treatment led to increased expression of activated β1 integrin (Fig. 6b) without increasing the total amount of either α5 or β1 (Fig. 6a), which are constitutively expressed on the apical (luminal) surface of endothelial cells (67). The finding of increased β1 activation was confirmed using two different anti-β1 antibodies (15/7 and HUTS-21; refs. 55, 59) and both ELISA and immunofluorescence techniques (Fig. 6b and Fig. 7, a and b). Furthermore, there was colocalization between HUTS-21 and 90.45 antibodies on the apical surface of MM-LDL–treated endothelial cells (Fig. 9c).

Further confirmation that activation of β1 can lead to apical expression of CS-1 FN in endothelial cells was provided by our studies with 8A2, a β1 integrin–activating antibody (54). We observed that treatment of endothelial cells with 8A2 increased monocyte-specific binding (Fig. 8a) and CS-1 but not VCAM-1 expression (Fig. 8b). Most definitively, 8A2-induced monocyte adhesion could be inhibited by treatment of monocytes with blocking VLA-4 antibodies (see Results) or by treatment of HAEC with the 90.45 antibody against CS-1 (Fig. 8c). These results with 8A2 strengthen our conclusion that increased CS-1 expression on the apical surface of MM-LDL–treated HAEC is due to increased deposition of apical FN by the activation of β1 integrin.

The present studies provide evidence that MM-LDL–mediated β1 activation and the formation of stable VLA-5/CS-1 complexes that bind monocytes involve several steps. Activation of integrins requires a conformational change that can be brought about directly by an activating antibody or indirectly by activation of G proteins, protein kinases, phosphatidylinositol turnover, and possibly protein synthesis (28). The pres-
dent studies suggest that MM-LDL–induced activation of β1 is indirect. MM-LDL treatment of HAEC caused a gradual increase in the expression of the activated β1 epitope over the course of four hours (Fig. 6b). This is in contrast to HAEC treated with MnCl₂, in which the expression of the HUTS-21 epitope occurred within 15 minutes. Our observations suggest that activation of β1 is necessary but not sufficient to cause monocyte binding. It is known that 8A2 activates β1 integrins, presumably by bringing about a conformational change, within 15 minutes (54). However, the induction of monocyte binding in response to 8A2 antibody took at least one hour (Fig. 8a). This suggests that some postreceptor events are involved in the process of monocyte binding in response to 8A2 treatment of HAEC. Ishida and colleagues (70) have shown that 8A2-induced FAK tyrosine phosphorylation in HAEC begins after at least 30 minutes and then progressively increases over the next two hours. Additionally, FAK phosphorylation leads to integrin clustering. It is known that the clustering of integrins involves the interaction of the cytoplasmic tails of integrins with several cytoskeletal proteins and extracellular matrix proteins on the outer side of the cells to constitute focal adhesions (71). Immunofluorescence confocal microscopy revealed a clustering of the activated β1 integrins on the apical surface in response to MM-LDL (Fig. 7b). We hypothesize that MM-LDL–induced integrin clustering is due to cytoskeletal interactions that lead to the patchlike distribution of CS-1. We have shown that treatment of endothelial cells with CHX before MM-LDL stimulation inhibited monocyte binding (37) and CS-1 expression (see Results). Thus, at least one step in the process leading to CS-1 expression and monocyte binding is mediated by new protein synthesis. The slow time course of β1 activation by MM-LDL and the slow induction of monocyte binding, may be explained by both the time required for inside-out activation and the time required for integrin clustering that facilitates FN deposition. The in vivo relevance of CS-1 to human atherosclerosis is suggested by the presence of CS-1 in the endothelium of human coronary lesions, although the exact location within the endothelium is not demonstrated by these studies (Fig. 11, c and e). A previous study (76) suggests a role for CS-1 FN in a number of other chronic inflammatory states. An increase in the amount of FN that is recognized by the 90.45 antibody has been identified in rheumatoid arthritis synovium, on the lumen of rheumatoid arthritic endothelium (47, 48, 58, 77), and in transplant coronary artery disease (48). Studies of chronic kidney inflammation have indicated that FN may also be involved in the inflammatory process (78–80). Antibodies to oxidized phospholipids are increased in animals and patients with atherosclerosis and a number of other chronic inflammatory conditions (81–83). On the basis of these observations, we hypothesize that oxidized phospholipids may contribute to chronic inflammation by activation of β1 dimerized to α5, leading to increased expression on the endothelial cell surface of CS-1 FN, a monocyte-specific ligand.

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