Rapid Communications

Blocking Very Late Antigen-4 Integrin Decreases Leukocyte Entry and Fatty Streak Formation in Mice Fed an Atherogenic Diet

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Abstract—Atherosclerotic lesion development is characterized by the recruitment of leukocytes, principally monocytes, to the vessel wall. Considerable interest has been focused on the adhesion molecule(s) involved in leukocyte/endothelial interactions. The goal of the present study was to determine the role of the very late antigen-4 (VLA-4) integrin/ligand interaction in fatty streak development using murine models. Because α4 null mice are not viable, a peptidomimetic was used to block VLA-4-mediated leukocyte binding. The ability of a synthetic peptidomimetic of connecting segment-1 (CS-1 peptide) to block the recruitment of leukocytes and the accumulation of lipid in the aortic sinus of either wild-type mice (strain C57BL/6J) or mice with a low-density lipoprotein null mutation (LDLR−/−) maintained on an atherogenic diet was assessed. The active (Ac) CS-1 peptide or scrambled (Sc) CS-1 peptide was delivered subcutaneously into mice using a mini osmotic pump. Mice were exposed to the peptide for 24 to 36 hours before the onset of the atherogenic diet. In C57BL/6J mice, leukocyte entry into the aortic sinus, as assessed by en face preparations, was inhibited by the active peptide (Ac=28±4, Sc=54±6 monocytes/valve; P=0.004). Additionally, frozen sections stained with Oil Red O were analyzed to assess lipid accumulation in the aortic sinus. C57BL/6J mice that received the (Ac) compound demonstrated significantly reduced lesion areas as compared with mice that received the (Sc) peptide (Ac=4887±4438 μm², Sc=15 009 ± 5619 μm²; P<0.0001). In a separate study, LDLR−/− mice were implanted with pumps containing either the (Ac) or (Sc) peptide before initiation of the atherogenic diet. Because LDLR−/− mice fed a chow diet displayed small lesions at 14 weeks, the effects of the peptide seen in these animals represented a change in early lipid accumulation rather than initiation. By using whole-mount preparations, the (Ac) but not the (Sc) peptide significantly reduced the area of lipid accumulation in the aortic sinus, resulting in an approximate 66% decrease. Plasma analysis from all studies revealed concentrations of peptide to be present at levels previously determined by in vitro analysis to block adhesion. (Ac) CS-1 peptide, which blocks VLA-4 on the leukocyte surface, is effective in reducing leukocyte recruitment and lipid accumulation in the aortic sinus. The present study provides in vivo evidence that the VLA-4 integrin plays an important role in the initiation of the atherosclerotic lesion and lipid accumulation, and it suggests a potential therapeutic strategy for this disease. (Circ Res. 1999;84:345–351.)

Key Words: atherosclerosis ■ monocyte ■ connecting segment-1 ■ fibronectin ■ α4β1

Previous studies have reported that monocyte but not neutrophil adhesion to the vascular endothelium is one of the first steps in the development of the fatty streak.1,2 Recent studies using mice that do not express monocyte activators including macrophage colony-stimulating factor,3,4 monocyte chemoattractant protein-1,5 and the monocyte chemoattractant protein-1 receptor6 have further defined the importance of monocyte recruitment to the endothelium in lesion areas, because all these studies reported significantly decreased lesion formation. Additionally, studies by Boisvert et al7 using an interleukin-8r (the GRO receptor) null mouse model demonstrate the importance of this monocyte activator in lesion formation. Monocytes can adhere to the endothelium by various integrins present on their surface. In vitro studies have demonstrated very late antigen-4 (VLA-4) to be a major ligand mediating firm adhesion of monocytes to the endothelium. Although T lymphocytes are also able to adhere via VLA-4, this interaction may not have a significant effect in fat-fed mice, because several different groups have reported that T lymphocytes did not effect atherosclerosis in fat-fed mice.8–10 In spite of the conclusions drawn from these lymphocyte studies, there are indications that lymphocytes may play a role in atherosclerosis under some conditions.8 Considered together, past studies on leukocyte, particularly...
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monocyte, adhesion suggest that VLA-4 may be important in atherosclerosis.

Two endothelial ligands for VLA-4 have been described: vascular cell adhesion molecule-1 (VCAM-1) and fibronectin containing the connecting segment-1 (CS-1) region. Expression of VCAM-1 has been shown to be increased in the aortic endothelium of rabbits given a high-fat diet and in the atherosclerotic lesions of certain mouse models of atherosclerosis. Recently, we have shown that CS-1 is increased in human coronary lesions, and thus it may be an important mediator in monocyte recruitment to the endothelium in vivo. The affinity of VLA-4 for VCAM-1 and CS-1 is comparable, although VLA-4 recognizes different sequences in VCAM-1 (QIDSPL) and CS-1 (LDV). Studies by several groups have reported the successful use of a CS-1 peptide in blocking cell-cell interactions in vitro and monocyte recruitment in vivo. Past studies demonstrate the ability of a CS-1 peptide to block binding of monocytes to VCAM-1 or CS-1. Therefore, we used CS-1 peptide infusion to determine the involvement of VLA-4 in fatty streak formation in an in vivo system.

The present study used C57BL/6J and low-density lipoprotein null mutation (LDLR−/−) mice fed a high-fat, high-cholesterol diet to examine the effect of VLA-4 on the development of early fatty streak lesions. The C57BL/6J mouse is susceptible to atherosclerosis and exhibits slow lesion development on a high-fat, high-cholesterol diet. In contrast, the LDLR−/− mouse developed more extensive lesions in a relatively short time period. Data obtained from both the C57BL/6J and the LDLR−/− mice substantiate an important role for VLA-4 in the development of the early atherosclerotic lesion.

Materials and Methods

In Vitro Studies
Rabbit and human aortic endothelial cells were isolated and cultured as previously described. Rabbit leukocytes were prepared using ficoll gradients and purified by adherence to plastic for 30 minutes. Human monocytes were prepared by a modified Recalde method. Preparation of minimally modified low-density lipoprotein (MM-LDL) and monocyte binding studies was performed as previously described.

Animals and Diets
Female C57BL/6J mice and LDLR−/− mice of a mixed genetic background (50% C57BL/6J and 50% 129/J) were obtained from Jackson Laboratories (Bar Harbor, Maine). Age-matched animals were housed together and fed standard chow (Purina No. 5001) until 10 weeks of age. At the onset of the study, the mice were individually housed and separated into the following groups for the study: (1) chow diet, no pump, (2) high-fat diet receiving active (Ac) CS-1 peptide, and (3) high-fat diet receiving scrambled (Sc) CS-1 peptide. Groups receiving high fat were maintained on an atherogenic diet for a relatively short time period. Data obtained from both the C57BL/6J and the LDLR−/− mice substantiate an important role for VLA-4 in the development of the early atherosclerotic lesion.

Preparation of the CS-1 Native and Scrambled Compounds
Peptides were obtained from Cytel Corporation (San Diego, Calif). Peptides were coded to perform blinded studies and were revealed after the completion of the studies. The (Ac) CS-1 compound is a 3-amino acid peptidomimetic corresponding to the C-terminal portion of the 25-aa CS-1 sequence, which inhibits VLA-4-mediated cell binding. The (Sc) CS-1 peptide consisted of identical amino acids; however, the sequence was scrambled. The peptides were diluted with sterile 1× PBS (with Ca and Mg) and loaded into the reservoir chamber of each mini osmotic pump (Alzet, Alza Corp). Peptides were delivered at 8 to 15 mg·kg⁻¹·d⁻¹ for 30 days.

Insertion of the Mini Osmotic Pumps
All procedures were carried out under the guidelines of the Animal Research Committee. Animals were anesthetized using Aerrane (isoflurane USP, Fort Dalge Animal Health, Fort Dalge, Iowa). The right lower quadrant of the animal was shaved with clippers and the skin cleansed with 70% ethanol. A 2-mm transverse incision was made, and a small subdermal pocket was created using a straight, long-nosed hemostat. The pump was inserted with the delivery pore located anterior toward the head of the animal, and the incision was secured using wound clips. Animals were implanted with the pumps 24 to 36 hours before the initiation of the atherogenic diet to enable the pump to begin peptide delivery into the bloodstream.

Plasma Lipid Levels and Peptide Analysis
A fasting blood draw was collected from the mice before the initiation of the study and on the day of their killing. Blood was collected by previously established methods. Total cholesterol, HDL, and triglyceride levels were determined by previously described enzymatic methods. Additional plasma samples were analyzed for levels of the CS-1 compounds by ELISA immune detection.

Generation of Aortic En Face Preparations
The aortic sinus and en face preparations were isolated and prepared using a variation on the method reported by Nakashima et al. To determine the effects of the (Ac) CS-1 peptide and (Sc) CS-1 peptide on leukocyte recruitment to the aortic sinus or on lipid accumulation, C57BL/6J or LDLR−/− mice fed either chow or a high-fat diet were killed, and the hearts were quickly perfused with 1× PBS containing 3 U/mL heparin. The heart and the ascending and descending aortas were removed and the following procedures were performed.

Leukocyte Identification in C57BL/6J Mice
The heart and ascending aorta were removed and fixed with 100% aceton. After 24 hours, the heart and aortic samples were rinsed 3 times with 1× PBS, and the tissue and aorta were trimmed until only the aortic sinus and aortic root remained. Care was taken to avoid contact with the aortic cusps during the manipulations, and excess fat and tissue on the back side of the aorta were also removed. The specimens were placed into a blocking solution of 1× PBS containing 3% BSA (3% APBS) for 1 hour at room temperature followed by rinsing 3 times with PBS and incubation with rat anti-human/mouse Mac-1 (CD11b/CD18) antibody (Boehringer-Mannheim) diluted in 3% APBS for 16 hours at 4°C. The next day, the tissue was again rinsed 3 times with 1× PBS and blocked for 1 hour at room temperature in a 1× PBS solution containing normal goat serum. The peroxidase-conjugated IgG anti-rat secondary antibody (Leinco Technologies) was diluted in 1× PBS/normol goat serum (Dako) solution and incubated for 2 hours at room temperature. The secondary antibody was recognized using an amino-9-ethyl carboxylate (AEC) kit (Biomedta). The aortic sinus was then placed onto a microscope slide and mounted using Crystal Mount (Biomedta). To determine the focal plane used to score the leukocytes, lipofuscin present on the valve leaflet served as the initial focal plane (Figure 1). It has been previously shown that lipofuscin develops on the flow-exposed surface of the valve leaflet across from the cusp where lipid, lipoproteins, and monocytes/macrophages have been detected. Once the level of the lipofuscin had been determined, the plane of focus was shifted to the area of the aortic sinus. Mac-1 leukocytes in 5 distinct regions in this area of the aortic valve were counted (using an eyepiece grid) under a light microscope using ×200 magnification.
Temperature. This high level of peptide was used to obtain peptide or no peptide, were incubated for 20 minutes at room temperature. The aortic sinuses were sectioned and stained with Oil Red O to detect lipids. Staining found below that of the lipofuscin. Figures 1 and 2. (Ac) CS-1 peptide reduces monocyte adhesion in vitro. Monocytes isolated from either humans or rabbits were pretreated with the (Ac) CS-1 peptidomimetic before adhesion to MM-LDL–treated ECs. Monocytes were resuspended in 1 mL of medium containing 500 μg/mL CS-1 peptide or no peptide and then incubated for 20 minutes at room temperature. Monocytes were then added to untreated ECs or to ECs treated for 4 hours with 200 μg/mL of MM-LDL. Treatment with peptide reduced binding both within and across species by 60% to 70% (Figure 2).

Active CS-1 Peptide Reduced Lipid Accumulation in the Aortic Sinus of C57BL/6J Mice

To assess the effect of the peptide on lesion initiation as measured by leucocyte recruitment to the aortic sinus, mini osmotic pumps containing either the (Ac) CS-1 peptide or (Sc) CS-1 peptide were implanted subcutaneously into C57BL/6J mice. The pumps were allowed to begin delivery of the peptides for 24 hours before the mice were subjected to 4 weeks of high-fat feeding. Leukocytes that were stained using a Mac-1 (CD18/CD11b) antibody were visualized as red, owing to AEC (Figure 3A through 3C). A single aortic cusp and valve from mice on a high-fat diet were sectioned and stained with Oil Red O for lipids as previously described. The number of sections that spanned the aortic sinus of each mouse was determined, and the size of the lesions present in these sections was measured by previously described methods.

Results

In Vitro Effects of the (Ac) CS-1 Peptide

The effect of pretreatment of monocytes with the CS-1 peptidomimetic and subsequent binding to MM-LDL–treated endothelial cells (ECs) was examined. Monocytes, resuspended in 1 mL of medium containing 500 μg/mL CS-1 peptide or no peptide, were incubated for 20 minutes at room temperature. This high level of peptide was used to obtain maximal effects. Monocytes were then added to untreated ECs or to ECs treated for 4 hours with 200 μg/mL of MM-LDL. Treatment with peptide reduced binding both within and across species by 60% to 70% (Figure 2).

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Statistical Analysis

Data were analyzed using the Statview 4.5 program. All P values were calculated using ANOVA and Fisher PLSD significance test.

Figure 1. Schematic diagram of the aortic sinus where leukocytes are scored. Lipofuscin forms on the valve leaflet across from where monocytes/macrophages accumulate. For frozen sections, the cusp region is round (A). In en face preparations, the valve leaflet is flattened against the back of the aortic sinus (B). Identifying the lipofuscin allowed for orientation of the plane of focus. Mac-1+ leukocytes were identified and counted in the plane of the aortic sinus found below that of the lipofuscin.

Figure 2. (Ac) CS-1 peptide reduces monocyte adhesion in vitro. Monocytes isolated from either humans or rabbits were pretreated with the (Ac) CS-1 peptidomimetic before adhesion to MM-LDL–treated ECs. Monocytes were resuspended in 1 mL of medium containing 500 μg/mL CS-1 peptide or no peptide and then incubated for 20 minutes at room temperature. Monocytes were then added to untreated ECs or to ECs treated for 4 hours with 200 μg/mL of MM-LDL at 37°C. Treatment with the peptide reduced binding both within and across species by 60% to 70% (P<0.001; n=12). Data are represented as mean±SD.

Measurement of Lipid Accumulation in the Aortic Sinus From LDLR−/− Mice

The aortic sinuses were isolated as described above; however, after removal of adventitial fat, the hearts and attached aortas were instead fixed with a 4% paraformaldehyde solution containing 5% sucrose for 24 hours at 4°C. After fixation, the hearts were thoroughly rinsed 3 times with 1× PBS before rinsing in 70% ethanol for 5 minutes at room temperature. The hearts were stained with a Sudan IV solution for 6 minutes before destaining with 80% ethanol to reduce background levels. The aortas were then rinsed with 1× PBS before mounting onto a slide with Crystal Mount. The area of valve covered by lipid was assessed using the NIH Image program. Aortic images (21 valves total) were captured by a video camera mounted to the top of a microscope. Both the lipid-laden regions and the total area of the valve itself were traced and compared. The area of valve covered by lipid was expressed as a percentage of the total area.

Measurement of Lipid Accumulation in Frozen Sections of C57BL/6J Aortic Sinus

Pumps were inserted into 8 C57BL/6J mice per group 24 hours before the initiation of the high-fat diet. After 4 weeks of high-fat feeding, the animals were killed, and the hearts were perfused and removed to be embedded into OCT compound (Tissue Tek). Hearts were sectioned and stained with Oil Red O for lipids as previously described. The number of sections that spanned the aortic sinus of each mouse was determined, and the size of the lesions present in these sections was measured by previously described methods.

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Reducing the Amount of Lipid Accumulation in the Aortic Sinus of LDLR<sup>−/−</sup> Mice

The ability of the (Ac) CS-1 peptide to decrease the lesion progression in LDLR<sup>−/−</sup> mice was determined by the amount of lipid accumulation in the aortic cusps of LDLR<sup>−/−</sup> mice after 3 weeks of high-fat feeding. En face preparations were made from LDLR<sup>−/−</sup> mice that were maintained on either a chow or high-fat diet; the latter group received either the (Ac) CS-1 peptide or (Sc) CS-1 peptide 24 to 36 hours before starting the high-fat diet. Figure 7A shows a single cusp from a mouse receiving the (Sc) CS-1 peptide. Areas of lipid stained a deep burgundy color on exposure to Sudan IV. Regions that stained deep burgundy were able to be visualized before staining under the dissecting microscope as areas of dense white-yellow accumulations, indicative of large lipid deposits (data not shown). Figure 7B was taken from a mouse receiving the (Ac) CS-1 peptide and the high-fat diet. NIH Image analysis was performed on the aortas, and the amount of lipid accumulation was expressed as the total area of valve covered with lipid. The aortas from animals that received the chow diet displayed a small amount of lipid accumulation (Figure 8). In conclusion, mice receiving the high-fat diet together with the (Ac) CS-1 peptide had 50% less lipid accumulation into the aortic sinus area as compared with mice receiving both the high-fat diet and the (Sc) CS-1 peptide (Figure 8).

Plasma Lipid and (Ac) CS-1 Peptide Levels

At the end of each experiment, fasting plasma was drawn from the mice for determination of lipid and peptide levels. Total cholesterol levels for both the C57BL/6J and LDLR<sup>−/−</sup> mice placed on the atherogenic diet were elevated as compared with group mates on the chow diet (Table). Differences in the total cholesterol levels from C57BL/6J mice were not significant between the high-fat groups treated with (Ac) CS-1 peptide or (Sc) CS-1 peptide. The LDLR<sup>−/−</sup> mice on the chow diet exhibited levels of total cholesterol that were significantly greater than the C57BL/6J mice on a chow diet (P<0.0001). This elevated cholesterol level in the LDLR<sup>−/−</sup> mice increased even further after high-fat feeding but also did not differ between peptide groups. Levels of

Figure 3. Representative aortic cusp from a mouse receiving either (Ac) CS-1 peptide or (Sc) CS-1 peptide stained for leukocytes using a Mac-1 (CD11b/CD18) antibody. Mini osmotic pumps containing either the (Ac) CS-1 peptide or (Sc) CS-1 peptide were implanted subcutaneously into the backs of C57BL/6J mice. Mice were then subjected to an atherogenic diet for 4 weeks. The aortic sinus was isolated, fixed with 100% acetone, and then stained with Mac-1 (CD11b/CD18) antibody for the presence of leukocytes. The Mac-1 antibody was visualized using AEC; therefore, the positively stained cells appear as red. Mac-1<sup>+</sup> leukocytes can be seen on the aortic sinus of mice receiving the (Sc) CS-1 peptide (arrow) (A). Mice receiving the (Ac) CS-1 peptide also contain Mac-1<sup>+</sup> leukocytes but are more difficult to visualize at this magnification (arrow) (B) (magnification ×40). With higher magnification of the aortic sinus from a mouse receiving the (Sc) CS-1 peptide, Mac-1<sup>+</sup> leukocytes appear as red (C) (magnification ×200).

Figure 4. (Ac) CS-1 peptide significantly reduces the amount of Mac-1<sup>+</sup> leukocytes bound to the aortic sinus of C57BL/6J mice maintained on an atherogenic diet. The aortic valve from each mouse was divided into 5 fields, and Mac-1<sup>+</sup> leukocytes were counted in each field. The (Ac) CS-1 peptide significantly reduced the levels of leukocytes bound to the aortic sinus of mice receiving the atherogenic diet. *P<0.0001; n=15 total number of valves for (Ac) CS-1 peptide and (Sc) CS-1 peptide. Data are represented as mean±SD.

Figure 5. Frozen sections from C57BL/6J mice stained with Oil Red O. Aortic sections from C57BL/6J mice that received either the (Ac) CS-1 peptide or (Sc) CS-1 peptide were stained for Oil Red O. Mice receiving the (Sc) CS-1 peptide exhibited concentrated lipid accumulation within the aortic sinus (A). The lipid accumulation from mice receiving the (Ac) CS-1 peptide stained with less intensity (B) (magnification ×100).

Figure 6. (Ac) CS-1 peptide significantly reduced the amount of lipid accumulated in the aortic sinus of C57BL/6J mice receiving an atherogenic diet. The area of lipid accumulation was calculated using an eyepiece counting grid. The lesion area of mice receiving the (Ac) CS-1 peptide was ∼2-fold less than that from mice receiving the (Sc) CS-1 peptide. *P<0.0001; n=8 animals for either (Ac) CS-1 peptide or (Sc) CS-1 peptide. Data are represented as mean±SD.
Figure 7. Aortic sinus of mice receiving either (Ac) CS-1 peptide or (Sc) CS-1 peptide were used to determine the effect of the peptides on lipids. The aortic sinuses of these mice were isolated in the same manner as that outlined above for the C57BL/6J mice. However, for this experiment, the en face preparation was fixed with a 4% paraformaldehyde/5% sucrose solution before staining with Sudan IV. Mice receiving the (Sc) CS-1 peptide demonstrated regions of lipid accumulation that appear as deep burgundy (arrows) (A). Mice receiving the (Ac) CS-1 peptide also contained some amount of lipid accumulation (arrow) (B) (magnification ×400).

HDL were found to be dramatically reduced in animals receiving the atherogenic diet as compared with chow for both strains of mice (Table). Peptide analysis in the blood demonstrated that the (Ac) CS-1 peptide was present in the bloodstream at concentrations (700 ± 243 ng/mL, η=1 μmol/L)26 that have been previously shown to block monocyte binding in vitro. In summary, treatment of mice with (Ac) CS-1 peptide did not alter elevated levels of plasma cholesterol in fat-fed mice. Therefore, the biological effects of the peptide are likely to be due to reduced leukocyte recruitment in vivo.

Discussion

The present study uses drug-delivery therapy to investigate the role of VLA-4 in vivo in the initiation and progression of the atherosclerotic lesion. In vivo studies to determine the role of other adhesion molecules in atherosclerosis have used gene targeting. However, this is not an option, because homozygous null mutations of the α4 and VCAM-1 genes have proven to be lethal.33–35 The use of peptides to block the interaction of leukocytes with the endothelium in vitro and in sites of inflammation in vivo has previously been reported by several groups.25,26 The in vitro blocking technique described in the present study demonstrated the ability of the (Ac) CS-1 peptide to reduce the adhesion of purified monocytes to either human or rabbit aortic ECs (Figure 2). Treatment with the (Ac) CS-1 peptide reduced binding of monocytes both within and across species by 60% to 70% (Figure 2). For in vivo studies, Wahl et al25 induced rheumatoid arthritis in a rat model that was found to be alleviated by treatment with the CS-1 peptide. In particular, CS-1 was effective at suppressing both acute and chronic inflammation, suggesting that the CS-1 peptide could influence both the initiation and progression of arthritis.25 Additionally, a CS-1 peptide similar to the compound in the present study has been reported to reduce the incidence of transplant atherosclerosis by attenuating intimal lesions in the coronary arteries of animals.36 Previous studies using monoclonal antibodies against α4 to investigate allergic asthma and inflammatory bowel disease suggest a role for α4 in the process of these inflammatory conditions.36 However, these results should be interpreted with caution, because some studies report side effects associated with the administration of an α4 antibody.

In the studies described above, peptides were delivered either intravenously or subcutaneously by injection. However, we chose to use a mini osmotic pump implanted subcutaneously into the animal. Different groups have successfully used osmotic pumps to deliver various therapeutic compounds.37,38 Use of mini osmotic pumps allows time-released delivery of the peptides and alleviates the need for daily injections, which can produce inflammation in addition to being labor-intensive. Furthermore, use of the pump reduced the possibility of dose variability from the experimenter or by receiving only a single large dose per day, given that the pump was designed to continuously deliver a set amount of peptide per day.

Monocytes have been shown to be the major VLA-4-containing cell type in atherosclerotic lesions,1,2 and previous studies have shown that neutrophils are not present in lesions.1 Our results demonstrate the ability of the (Ac) CS-1 peptide to reduce the recruitment of Mac-1⁺ leukocytes in vivo; the majority of these cells most likely are monocytes. The (Ac) CS-1 peptide blocks Mac-1⁺ leukocyte recruitment to the endothelium by inhibiting VLA-4-mediated binding of
leukocytes to their counter-receptors. Because T-cell lymphocytes are also able to adhere via VLA-4, it is possible that the (Ac) CS-1 peptide could inhibit their recruitment. However, studies by 2 separate groups using the apolipoprotein E−/− mouse as an atherosclerosis model independently scored either T-cell lymphocytes or macrophages.6 Comparing the 2 studies revealed that apolipoprotein E−/− lesions had a 1:500 lymphocyte to macrophage ratio. Studies by other investigators failed to identify T lymphocytes in mouse lesions at 15 weeks of feeding.13 These studies suggest that monocyte/macrophages represent at least 95% of the leukocytes in mice lesions. On the basis of these observations, we believe that the majority of leukocytes that stained positively with the Mac-1 antibody and those that are blocked by the (Ac) CS-1 peptide are monocyte/macrophages.

The (Ac) CS-1 peptide was effective at reducing lesion size in both C57BL/6J and LDLR−/− mouse models. Lipid analysis of frozen sections from C57BL/6J mice demonstrated that the (Ac) CS-1 peptide caused a 66% reduction in the size of lesions induced by high-fat feeding (Figure 6). Although LDLR−/− mice have been reported not to develop lesions on a chow diet, we and other investigators who have worked with the LDLR−/− mice have observed these mice to demonstrate a mild degree of spontaneous lesion development (J.H. Qiao, MD, oral communication, May 1996). En face sections taken from LDLR−/− mice demonstrated the presence of baseline lipid accumulation in mice receiving the chow diet. Mice receiving the (Sc) CS-1 peptide had a 4-fold increase in lesion size on a high-fat diet as compared with mice on a chow diet. The (Ac) CS-1 peptide reduced this increase by 50% (Figure 8). Thus, the peptide blocked the initiation of lipid accumulation in small lesions. Previous studies have shown that in the fatty streak, lipids mainly accumulated in macrophage foam cells whose entry is inhibited by the peptide. Therefore, the present study suggests that leukocyte VLA-4 binding of CS-1 is responsible for the inhibition of lesion development.

The present study used 2 different methods to analyze diet-induced atherosclerosis. En face or whole-mount preparations of the aortic sinus were initially used to assess the number of leukocytes bound. The focus of the present study remained on the aortic sinus, because this region has been reported to be the primary site of predilection in several strains of atherosclerosis mouse models including C57BL/6J.17 The same mouse model and technique were also used to determine levels of lipid accumulation. En face preparations have been reported to be less variable with less skewness as compared with traditional histological sections.39 Other advantages to this method include speed of generating samples for analysis. Additionally, en face preparations provide a better orientation of the tissue specimen. However, using this method does not allow for determination of the depth of the lesion. Furthermore, background exogenous tissue may cause difficulty with the analysis by increasing the opacity of the tissue thereby obscuring areas of interest. Therefore, histological sections were also examined to generate a more 3-dimensional image of the lesion. The difference in lesion size was greater in the animals when frozen sections were used, but statistically significant differences were seen with both methods.

The partial inhibition of lesion formation seen in the present study is similar to the effects in other studies targeting single adhesion ligands. Mice with single mutations in CD18, intercellular adhesion molecule-1, and P-selectin exhibited 47%, 63%, and 63% reductions in lesion area, respectively.40 There are several possible explanations for the partial inhibition of leukocytes adhering to the aortic sinus in animals receiving the active CS-1 peptide. The peptide levels used may not be great enough to cause complete saturation of the VLA-4 sites on the surface of leukocytes in vivo. However, previous studies by others showed an IC50 of 0.2 to 0.5 μmol/L in vitro, a concentration that was half of what we observed in the mouse plasma.18,26 Another possibility is that leukocytes may be able to use more than one surface integrin to adhere to the endothelium. Issekutz41 has previously reported that there are 3 leukocyte integrins responsible for in vitro adhesion and in vivo migration to sites of inflammation: Mac-1, lymphocyte function-associated antigen-1 (LFA-1), and VLA-4. Blocking studies demonstrated that, of these 3, LFA-1 and VLA-4 together were mostly responsible for the adhesion and migration of leukocytes. Complete inhibition of monocytes was only achieved after blocking against all 3 integrins.41 Furthermore, Issekutz41 reported that the initiating inflammatory stimulus is able to modify leukocyte integrin use. These studies highlight the importance of VLA-4 in monocyte recruitment in atherosclerosis; however, they cannot exclude the possibility of alternative ligands and/or pathways.

In summary, our data demonstrate the importance of VLA-4 in the early stages of Mac-1− leukocyte adhesion and lipid accumulation that occur in response to a high-fat diet. The present study has focused on VLA-4–mediated Mac-1− leukocyte entry into early fatty streak lesions and the effect of a CS-1 peptidomimetic on this interaction. The more recent study reported by Patel et al42 investigated the role of VLA-4 in advanced atherosclerosis using an anti-α4 antibody. However, the study of Patel et al used labeled macrophages rather than the natural monocyte population. The present study together with the study of Patel et al supports the importance of VLA-4 in regulating leukocyte entry into both early and advanced lesions where they may contribute to plaque rupture. Furthermore, our results suggest that VLA-4 peptidomimetics may be useful in limiting Mac-1− leukocyte entry into atherosclerotic lesions. Studies to determine the subunit recognized by monoclonal antibodies indicate that antibodies against epitope A of α4 partially block the interaction between VLA-4 and fibronectin without inhibiting VCAM-1 adhesion.43 This suggests the possibility of generating an anti-α4 peptide that would be specific for blocking VLA-4–mediated adhesion to CS-1 only.

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References


Blocking Very Late Antigen-4 Integrin Decreases Leukocyte Entry and Fatty Streak Formation in Mice Fed an Atherogenic Diet

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