An Atherogenic Diet Rapidly Induces VCAM-1, a Cytokine-Regulatable Mononuclear Leukocyte Adhesion Molecule, in Rabbit Aortic Endothelium

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Accumulation of monocyte-derived foam cells in focal areas of the arterial intima is a key step in early atherogenesis. We investigated the expression of vascular cell adhesion molecule-1 (VCAM-1), a mononuclear leukocyte adhesion molecule, in the arterial endothelium during the early phases of diet-induced atherogenesis in rabbits in vivo and the regulation of VCAM-1 expression by cytokines in rabbit aortic organoid cultures in vitro. Rabbits were fed either an atherogenic diet (containing 0.3% cholesterol, 9% coconut oil, and 1% corn oil) or an isocaloric control diet (10% corn oil) for 4 days or 1, 3, 6, or 13 weeks. The endothelium in the ascending aorta focally expressed VCAM-1 after only 1 week on the atherogenic diet but before the first appearance of intimal macrophages, as judged by immunohistochemical staining of serial sections. The rabbits that consumed the atherogenic diet for 3 weeks or longer developed lesions in the intima composed of macrophages bearing class II major histocompatibility antigen (MHC-II). Endothelial cells continued to focally express VCAM-1 at sites of MHC-II-positive intimal macrophages for up to 13 weeks. The ascending aortas of control rabbits lacked VCAM-1 or MHC-II-positive endothelium or macrophages at all times studied. These observations demonstrate the focal activation of arterial endothelium as early as 7 days after initiation of an atherogenic diet (at serum cholesterol levels of 308±57 mg/dl). In organoid cultures of rabbit thoracic aortas, Gram-negative bacterial lipopolysaccharide or the cytokines interleukin (IL)-1α, tumor necrosis factor-α, and IL-4, as well as interferon gamma, induced VCAM-1 expression in the endothelium. Our results suggest that the activation of cytokine-inducible endothelial functions, such as expression of VCAM-1, may participate in the initiation of diet-induced atherosclerosis in rabbits.

KEY WORDS • atherosclerosis • lesion initiation • monocyte adhesion • VCAM-1 • cytokines • immunohistochemistry • rabbits

Local adherence of circulating monocytes to the vascular endothelial lining is one of the earliest morphological alterations in the arteries of animals after initiation of an atherogenic diet.1-3 Adherent monocytes subsequently migrate into the intima, accumulate lipids, and become foam cells. Monocyte-derived foam cells are also a major component of the early atherosclerotic lesion in human arteries.4-6 Alterations in adhesive properties of the vascular endothelial surface due to the induction of endothelial-leukocyte adhesion molecules (ELAMs) might contribute to monocyte recruitment in the arteries of hypercholesterolemic animals. Among the several ELAMs identified and structurally characterized to date, vascular cell adhesion molecule-1 (VCAM-1) holds particular interest in regard to the initiation of macrophage-rich fatty streak lesions because of its functional specificity and pattern of expression.

VCAM-1, a member of the immunoglobulin (Ig) gene superfamily, is a mononuclear leukocyte-selective adhesion molecule expressed in cultured human vascular endothelial cells (ECs) after activation by lipopolysaccharide (LPS) and certain cytokines, such as interleukin-1 (IL-1), tumor necrosis factor (TNF), and IL-4.7-8 This inducible EC surface molecule mediates intercellular adhesion via interaction with its counterreceptor, from the Vascular Medicine and Atherosclerosis Unit (H.L., P.L.), Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, the US Department of Agriculture, Human Nutrition Research Center on Aging at Tufts University (H.L.); and the Vascular Research Division (M.I.C., M.A.G.Jr.), Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston.

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very late activation antigen-4 (VLA-4), a β2-integrin expressed by monocytes, lymphocytes, basophils, eosinophils, and certain tumor cells, but not neutrophils. 9,10 VCAM-1 expression increases in the endothelium of postcapillary venules at sites of inflammation and in cardiac allografts undergoing rejection. 11 Nonvascular cells can also express VCAM-1, including dendritic cells in lymphoid tissues and skin, monocyte-derived cells in the liver and spleen, and some epithelial cells in the kidney. 10,12,13 Recent studies have disclosed that the endothelium that overlies foam cell lesions of aortas in hyperlipidemic rabbits focally and selectively expresses VCAM-1, as detected with the monoclonal antibody (mAb) Rbl/9. 14 The restricted expression pattern of rabbit VCAM-1 indicated that the expression of this molecule might result from endothelial activation and might participate in local endothelial–monocyte interaction during the initiation of atherosclerosis. However, to date it has not been documented that cells within human atherosclerotic plaques express VCAM-1.

Human atherogenesis generally involves many years of silent evolution, thus limiting our ability to study its initial phases. Animal experiments can provide insight into the pathogenesis of this complex and prolonged process by permitting precise control of atherogenic variables and sampling over a range of time points. The current study aimed to evaluate the time course of VCAM-1 induction in vivo in response to hypercholesterolemia and the temporal and spatial relation between VCAM-1 expression and macrophage-derived foam cell lesion formation. Furthermore, the effect of a variety of pathophysiologically relevant stimuli on endothelial VCAM-1 expression was examined ex vivo using an organoid culture system.

**Methods**

**Animals**

Fifty male New Zealand White, Pasteurella-free rabbits weighing approximately 2.5 kg were purchased from Millbrook Farms, Amherst, Mass. Animals were housed at an animal facility accredited by the American Association for the Accreditation of Laboratory Animal Care, and experiments were conducted in accordance with National Institutes of Health guidelines. Rabbits were randomly assigned to two dietary groups. One group of rabbits consumed a semipurified atherogenic diet (No. D30268, custom prepared by Research Diets Inc., New Brunswick, N.J., according to our formulation), 15 which contained 0.3% cholesterol, 9% partially hydrogenated coconut oil (a source of C10–12 saturated fatty acids), and 1% corn oil (a source of polyunsaturated fatty acid). The other group consumed an isocaloric control diet, also semipurified from Millbrook Farms, Amherst, Mass. Animals were housed at an animal facility accredited by the American Association for the Accreditation of Laboratory Animal Care, and experiments were conducted in accordance with National Institutes of Health guidelines. Rabbits were randomly assigned to two dietary groups. One group of rabbits consumed a semipurified atherogenic diet (No. D30268, custom prepared by Research Diets Inc., New Brunswick, N.J., according to our formulation), 15 which contained 0.3% cholesterol, 9% partially hydrogenated coconut oil (a source of C10–12 saturated fatty acids), and 1% corn oil (a source of polyunsaturated fatty acid). The other group consumed an isocaloric control diet, also semipurified (No. D30003, Research Diets Inc.), 16 which contained 10% corn oil without a cholesterol supplement. The use of semipurified diets permits more rigorous control of possible variations in diet composition. The use of isocaloric diets with the equivalent energy density also avoids confounding variables in dietary composition other than the intended changes in fat and cholesterol composition.

**Animal Study Protocol**

The rabbits were housed in individual cages. Diet and water were provided ad libitum. Food intake was recorded daily, and body weight was monitored weekly. Blood was drawn from the marginal ear vein into EDTA-containing tubes at 0, 1, 3, 6, and 13 weeks. Plasma cholesterol was measured enzymatically using a Roche Cobas Mira random-access analyzer. Total triglycerides were also measured enzymatically using a Roche Cobas Fara II centrifugal analyzer. Plasma aspartate-leucine transferase (ALT), aspartate-serine transferase (AST), and alkaline phosphatase (ALP) levels were monitored as indicators of liver function (elevated levels of ALT and AST indicate hepatocellular damage, whereas elevated ALP level suggests biliary dysfunction). Groups of four (in the control group) or five (in the atherogenic diet group) rabbits were heparinized (2,000 units/rabbit i.v.) and killed by intravenous injection of a lethal dose of pentobarbital (Beuthanasia-D Special, Schering Corp., 0.7 ml/rabbit) after 4 days or 1, 3, 6, or 13 weeks on their respective diets. Thoracic aortas were dissected and immersed in Dulbecco's phosphate-buffered saline, and the adventitia was removed. To achieve consistency in sampling site, a 5-mm segment of the ascending thoracic aorta was obtained 1 mm above the aortic valve, snap-frozen in isopentane, and cooled in liquid nitrogen. Segments from various parts of the descending thoracic or abdominal aorta were also obtained and prepared for immunohistochemical study.

**Organ Culture Studies**

Thoracic aortas were removed from four rabbits (fed normal rabbit chow and weighing about 3 kg) killed by injection of pentobarbital. After rinsing with phosphate-buffered saline and gentle removal of the adventitia, aortas were cut into rings about 5 mm long. These aortic rings were placed in six-well tissue culture plates (Falcon) containing Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. LPS (from Escherichia coli, 055: B5, 1 µg/ml, Sigma), rabbit IL-1α (100 ng/ml, Glaxo), rabbit interferon gamma (IFN-γ, 1,000 units/ml, Genentech), human TNF-α (2 ng/ml, Endogen), and IL-4 (2 µg/ml, Collaborative Research) were added to the organ culture medium, and the plates were placed on a Bellco low-profile rocker (Bellco, Vineland, N.J.) in a tissue culture incubator at 37°C for 2, 4, 8, 16, or 24 hours. At the end of each time point, the aortic rings were washed in phosphate-buffered saline and snap-frozen in liquid nitrogen.

**Antibodies**

The following mAbs were used for immunohistochemical analysis: mAb Rbl/9 (mouse IgG1, hybridoma supernatant), which recognizes 118- and 98-kd forms of rabbit VCAM-114; mAb RAM-11 (mouse IgG1, purchased from Enzo Diagnostic Inc., New York), which recognizes muscle-specific α-actin.16 The preservation of aortic ECs in frozen section was

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Effects of Experimental Diets on Plasma Cholesterol, ALT and AST transaminases and ALP remained stable throughout the experiment (data not shown). Thus, as previously reported, the moderate level of cholesterol supplementation (0.3%) used in this experiment did not affect general nutritional status, nor did it cause liver damage that is often associated with atherogenic diets that contain higher levels of cholesterol.

**VCAM-1 Expression and Cell Identification**

Endothelial VCAM-1 expression was analyzed immunohistochemically at identical sites in the ascending aortas of rabbits fed either the atherogenic or control diet for 4 days to 13 weeks. The ascending aortas of control rabbits at all times studied lacked focal endothelial VCAM-1 expression (Figure 2a), as did the aortas of rabbits that had consumed the atherogenic diet for 4 days (data not shown). The aortic intimas of the rabbits fed either the control diet or the cholesterol-enriched diet for 4 days also consistently lacked macrophages or MHC-II-positive cells (data not shown). The typical punctate staining pattern of vWF (Figure 2b) verified the preservation of an intact endothelial lining in these specimens.

Sections from three of five rabbits that consumed the atherogenic diet for 6 weeks exhibited focal endothelial VCAM-1 expression (Figure 2c). At this time, the aortas of all rabbits lacked intimal macrophages (Figure 2d) and MHC-II-positive cells (data not shown), as determined by staining of serial sections. After 3 weeks on the atherogenic diet (the next time point sampled), four of five rabbits exhibited focal areas of VCAM-1-bearing ECs in the aortic intima (Figure 3a). Examination of the adjacent cross sections at this time point revealed frequent macrophages (Figure 3b) localized in the intima underlying the VCAM-1-bearing ECs, but not in the intima underneath the VCAM-1-negative endothelium. These intimal macrophages always stained positively for MHC-II antigen (data not shown). The aortas of all five rabbits that consumed the atherogenic diet for 6 weeks contained areas of thickened intima with foam cell lesions. The endothelium overlying these foam cell lesions frequently expressed VCAM-1 (Figure 3c) while ECs in lesion-free areas typically lacked VCAM-1 (Figure 3d). At 13 weeks, foam cell lesions covered almost the entire luminal surface of the ascending aortas in all five rabbits fed the atherogenic diet. Most of the ECs overlying these foam cell lesions expressed VCAM-1 (not shown).
We also examined some random sections from thoracic and abdominal aortas for VCAM-1 expression and cell identification markers (data not shown). We did not detect VCAM-1 expression in any of these sections from rabbits fed the control diet or the atherogenic diet for 1 week or less. After the rabbits had been on the atherogenic diet for 3 or more weeks, we observed occasional but not widespread or consistent lesions with VCAM-1–positive ECs in the descending thoracic or abdominal aorta.

**Effects of Cytokines on VCAM-1 Expression in Rabbit Aortic Organoid Cultures**

Rings of rabbit thoracic aortas incubated in the control medium for 2–24 hours showed no or only occasional endothelial VCAM-1 expression (Figure 4a). Staining for vWF showed preservation of the intact endothelium on aortic organoid cultures under all treatments (Figure 4a, insert), except after incubation with LPS for more than 8 hours, a condition that produced degeneration of the intimal endothelium (not shown). LPS (Figure 4b) markedly augmented endothelial VCAM-1 expression at 8 hours in aortic rings from all four rabbits. Rabbit IL-1α (Figure 4c), IFN-γ (Figure 4d), human TNF-α (Figure 4e), and human IL-4 (Figure 4f) also stimulated endothelial VCAM-1 expression at 24 hours in aortic rings from all four rabbits.

**Discussion**

This study examined the temporal and spatial relation of endothelial VCAM-1 expression to MHC-II expression and monocyte/macrophage accumulation in the intima of the rabbit ascending aorta during the earliest stages of diet-induced hypercholesterolemia. To avoid the lack of nutrient definition and the variability of commercial rabbit chow, we used semisynthetic diets for this study. Rabbits fed diets containing 1–2% cholesterol soon develop hepatic disease and fail to thrive, as suggested by impaired growth. In this study, a diet supplemented with 0.3% cholesterol produced fatty streaks in the ascending aorta within 3–6 weeks, yet avoided the hepatotoxicity and growth-inhibitory effect of atherogenic diets containing higher levels of cholesterol.

In animals with diet-induced or genetically determined hyperlipidemia, the earliest morphological
changes in arteries include focal adherence of mononuclear leukocytes to the endothelium and accumulation of monocyte-derived foam cells in the intima. Lipid-laden macrophages are a major component of human atheromas and of atherosclerotic lesions in hyperlipidemic animals. During the last eight decades, many studies have investigated the effects of atherogenic diets on plasma lipoproteins and the morphology or biochemistry of arterial lesions. Yet, the mechanisms that link diet-induced hyperlipidemia to the evolution of cellular changes in the arterial wall remain poorly understood. The localized nature of the mononuclear leukocyte–EC interaction leads to the hypothesis that this relation might result from focal changes in the adhesive properties of the endothelial surface, including the expression of molecules such as VCAM-1, that selectively promote binding of mononuclear leukocytes to the vascular endothelium.

The localization of VCAM-1 in the endothelium overlying aortic foam cell lesions of dietary and heritable hypercholesterolemic rabbits suggests that this molecule may participate in monocyte recruitment during early atherogenesis. However, VCAM-1 expression might conceivably result from macrophage accumulation because activated macrophages can produce many of the cytokines known to stimulate endothelial VCAM-1 expression. This study examined the temporal and spatial relation between VCAM-1 expression and macrophage accumulation to further define the role of VCAM-1 in early atherogenesis by testing whether expression of this molecule precedes or follows monocyte recruitment. Because formation of human atheromas usually requires many years, it will prove difficult to examine the expression of adhesion molecules during the earliest phases of atherogenesis, particularly since postmortem changes in the luminal endothelium are virtually inevitable under the usual conditions of access to early human lesions (e.g., at autopsy). Hence, we approached this issue in an experimental preparation that permits serial sampling under controlled conditions. We selected the cholesterol-fed rabbit to study this question because of the relatively rapid formation, in predictable locations, of lesions that share features of human fatty streaks. The ascending
aorta (1–5 mm above the aortic valve) was evaluated to ensure consistency of sampling. This segment of aorta has a relatively lower susceptibility to foam cell lesion formation than does the aortic arch and regions downstream of the ostia of aortic branches, as determined by oil red O staining.

The ascending aortic endothelium focally expressed VCAM-1 very early after initiation of an atherogenic diet (absent at 4 days but often present after only 1 week). Intimal macrophage accumulation and expression of MHC-II antigen, a marker of macrophage or smooth muscle cell activation,27 occurred later (1–3 weeks) and only in areas where the endothelium expressed VCAM-1. Most of the MHC-II–expressing cells seemed to be macrophages, as judged by comparing the staining pattern of MHC-II and macrophages in serial sections. Hansson et al28 also reported that macrophages in atherosclerotic lesions express MHC-II. The expression of VCAM-1 documented here establishes endothelial activation as an early feature in the formation of arterial lesions in response to an atherogenic diet. The temporal and spatial relations between expression of VCAM-1 and macrophage accumulation further support a role for VCAM-1 in enhanced endothelial-leukocyte interaction and monocyte recruitment during the initiation of diet-induced atherosclerotic lesions.

Cytokines that are locally elaborated by vascular cells or leukocytes might induce endothelial expression of adhesion molecules.13,26–29 IL-1, TNF, IL-4, or LPS promote VCAM-1 (as well as ELAM-1 and intercellular adhesion molecule-1 [ICAM-1]) expression by cultured human vascular ECs.30,31 However, IFN-γ, a product of activated T or natural killer cells, stimulates expression of ELAM-1 and ICAM-1 but not VCAM-1 in cultured human ECs.13,30,31 We used short-term aortic organoid cultures to define the range of possible mediators of diet-induced VCAM-1 expression in the rabbit arterial endothelium. The aortic organoid cultures enabled us to study endothelial functions in the context of their...
normal matrix and without possible alterations (e.g., loss of cytokine reactions) due to propagation in cell culture. We demonstrated that the noncytokine stimulus LPS or the cytokines IL-1α, TNF-α, and IL-4, as well as IFN-γ, induced VCAM-1 expression in the rabbit endothelium in these organoid culture preparations. The effect of IFN-γ on endothelial VCAM-1 expression is of particular interest because it suggests the potential involvement of T cells in regulating this mononuclear-leukocyte specific adhesion molecule. Hansson et al. have identified T cells adhering to the aortic surface and accumulating within the intima during the early stages of diet-induced atherosclerosis in rabbits. A recent study shows that VLA-4-VCAM-1 and lymphocyte function associated molecule-1-ICAM-1 interactions mediate costimulation of T cells. Such newly defined, specialized accessory functions of these integrin pathways and the identification of T cells in the aortic lesion, together with our finding that a product of activated T cells strongly induces endothelial VCAM-1 expression, imply an important role for T-cell activation during early atherogenesis.

Possible triggers for local cytokine generation might include hydrodynamically altered cell functions predisposing to intimal lipid accumulation and modification. Lyosphosphatidylcholine, a component of oxidatively modified low density lipoprotein, also can promote VCAM-1 mRNA and surface protein expression, as well as increasing the role of ICAM-1 in cultured ECs. Thus, a product of lipoproteins modified in situ might induce endothelial surface VCAM-1 expression directly or via autocrine or paracrine cytokine production.

Monocyte recruitment during development of an atherosclerotic lesion likely depends on a number of factors other than VCAM-1. One candidate that might participate in monocyte recruitment is ICAM-1, an adhesion molecule expressed by the endothelium that promotes the adhesion of monocytes, neutrophils, and lymphocytes via binding of ICAM-1 to the surface membrane β₂-integrin molecule, LFA-1, on these leukocytes. A recent study demonstrated increased endothelial ICAM-1 expression in human atherosclerotic lesions, further supporting the role of ICAM-1 in early atherogenesis. Other factors that might contribute to monocyte recruitment include leukocyte adherence molecule-1 or locally elaborated chemoattractants such as monocyte chemotactic protein-1. Cooperation between adhesion molecules alone or in concert with the effect of chemoattractants may contribute to the initiation and development of macrophage-rich atherosclerotic lesions.

The present results focus future research in three directions. First, by knowing the sequence of events in this well-defined and reproducible in vivo preparation, we can now seek evidence of local cytokine expression at an appropriate time and place to learn more about the signaling events that yield local prelesional VCAM-1 expression. Second, our results identify VCAM-1 as a likely target for interventions (e.g., the use of an anti–VCAM-1 antibody or other strategies in vivo) to dissect its functional role in leukocyte recruitment in atherogenesis. Third, our study suggests that the T-cell activation product IFN-γ may play an important role in regulating endothelial VCAM-1 expression and thus points out the need for further study of the role of this cytokine in early atherogenesis in vivo.

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