Increased Expression In Vivo of VCAM-1 and E-Selectin by the Aortic Endothelium of Normolipemic and Hyperlipemic Diabetic Rabbits

Mary Richardson, Steven J. Hadcock, Marnie DeReske, Myron I. Cybulsky

Abstract Atherosclerosis is enhanced in humans with diabetes mellitus, but the mechanism(s) involved remains unclear. Increased leukocyte-endothelium interaction may be involved, since mononuclear leukocyte adherence to the endothelium is an early event in both experimental atherosclerosis and alloxan-induced diabetes in rabbits. In situ immunohistochemistry was used in en face Haéutchen endothelial preparations to identify endothelial cells that stained with antibodies to endothelial leukocyte adhesion molecules (vascular cell adhesion molecule-1 [VCAM-1] and E-selectin), and the number of stained cells per 10,000 cells was determined. Preparations from aortas of diabetic normolipemic and egg yolk diet-induced hyperlipemic diabetic rabbits were compared with those from normolipemic animals on similar diets. Cross sections of the vessel wall were stained with oil red O and antibodies to VCAM-1, E-selectin, and RAM-11-positive macrophages. After 4 weeks of hyperlipemia the frequency of cells expressing VCAM-1 or E-selectin was significantly increased compared with normolipemic controls; this frequency was further increased in the aortas of hyperlipemic diabetic rabbits. VCAM-1 and E-selectin expression was more frequent in normolipemic diabetic rabbit aortas than in hyperlipemic, normolipemic vessels. The potentiation of expression of these adhesion molecules in diabetic animals may provide part of the explanation for the enhanced atherosclerosis associated with diabetes mellitus. (Arterioscler Thromb. 1994;14:760-769.)

Key Words: vascular cell adhesion molecule-1 • E-selectin • endothelium • alloxan diabetes • atherosclerosis • immunohistochemistry

Patients with diabetes mellitus develop premature atherosclerosis that is more severe and occurs more frequently than in similar nondiabetic populations, but the precise mechanisms involved in the enhanced atherosclerosis are not understood. The increased severity may be related to a potentiated recruitment of white blood cells to the endothelial surface. The adherence of mononuclear leukocytes to the arterial endothelium is one of the earliest detectable events in diet-induced experimental atherosclerosis in a number of species, including primates and rabbits. In the first 6 months of alloxan-induced diabetes in normolipemic rabbits, we have observed a progressive adherence of mononuclear leukocytes to aortic endothelium and recruitment to the subendothelial space. The molecular basis for the adherence of leukocytes to the endothelium has been extensively examined in vitro (for reviews, see References 8 through 12). It is well established that endothelial cells stimulated by a variety of cytokines will express adhesion molecules, including E-selectin (endothelial leukocyte adhesion molecule-1) and vascular cell adhesion molecule-1 (VCAM-1), and leukocytes express specific counterreceptors or ligands. Endothelial cell leukocyte adhesion molecules have been demonstrated in vivo by immunohistochemical studies in which E-selectin and VCAM-1 have been identified in biopsy material from a variety of human inflammatory conditions, including rheumatoid and osteoarthritic synovial tissues and reactive lymph nodes. The possible involvement of endothelial leukocyte adhesion molecules in arterial disease has more recently been recognized. VCAM-1, a transmembrane protein and member of the immunoglobulin gene superfamily, supports the adhesion of monocytes and lymphocytes but not neutrophils to endothelium, and it has been identified by immunohistochemical studies in endothelial cells and macrophages in aortic endothelium over foam cell–rich lesions of Watanabe heritable hyperlipidemic and cholesterolemia-fed rabbits and in endothelium over atherosclerotic lesions in human coronary arteries. Intercellular adhesion molecule-1 (ICAM-1), also a transmembrane protein member of the immunoglobulin gene family, supports the adhesion of all leukocytes and is expressed on endothelial cells and smooth muscle cells in human atherosclerotic lesions. Increased expression of E-selectin, which supports the adhesion of neutrophils, certain T lymphocytes, and monocytes to endothelium in vitro, has recently been detected at low levels in human atherosclerotic lesions. In vivo studies indicate that at sites of inflammation E-selectin is confined to the endothelium and in rat models of skin and lung injury it is involved in the emigration of neutrophils.

In this study we quantified and compared the extent of expression of VCAM-1 and E-selectin in aortas of rabbits with alloxan-induced diabetes in which hyperlipemia was also induced by an egg yolk–supplemented diet. Diabetic normolipemic, normoglycemic hyperlipemic, and normolipemic normoglycemic rabbit aortas were examined in the same way. In situ immunohistochemistry in en face preparations of aortic endothe-
lium was used, and the spatial relation between adhesion molecule expression and intimal macrophage accumulation was evaluated from cross sections of the vessel wall. We found increased expression of VCAM-1 and to a lesser extent E-selectin in alloxan-induced diabetic rabbits; this expression was further increased by the superimposed diet-induced hyperlipemia.

Methods

Materials

The monoclonal antibody (mAb) Rb1/9 to VCAM-1 was provided by Drs M. Cybulsky and M. Gimbrone, Brigham and Women's Hospital, Boston, Mass. The antibody to E-selectin, mAb 14G2, was the gift of Dr B. Wolitsky, Hoffmann-La Roche, Nutley, NJ. RAM-11 anti-rabbit macrophage mAb was the gift of Dr Alan Gown, Seattle, Wash. Goat anti-human von Willebrand factor (vWF) was purchased from Atlantic Antibodies.

The mAbs were visualized by using biotinylated horse antibodies to murine immunoglobulin (Ig) G and avidin-biotin peroxidase complexes (Vector Laboratories) or with the alkaline phosphatase-linked universal mouse detection system (AS/AP Immunostaining Kit; Bio/Can Scientific). The anti-vWF was visualized by using biotinylated rabbit anti-goat IgG and the Vectorstain ABC Kit (Vector Laboratories).

Animal Preparation

All animal procedures were in accordance with the recommendations of the Canadian Council for Animal Care. Male New Zealand White rabbits with an initial body weight of 3.5 to 3.8 kg were used throughout the study. Normolipemic animals were maintained on standard laboratory chow. Hyperlipemia was induced by supplementing the standard laboratory chow with 15% by weight dried egg yolk (Dominion Egg Ltd). This diet was fed ad libitum and was well tolerated by the animals. All animals had access to water at all times.

Diabetes mellitus was induced by injection of alloxan as described. Briefly, rabbits were selected at random and injected intravenously via a marginal ear vein with alloxan (65 mg/kg) freshly dissolved in sterile saline (50 mg/mL). The remaining animals, which formed the control group, were injected with saline alone. Six hours after the alloxan injection the rabbits received intraperitoneal glucose to counteract the hypoglycemia caused by insulin release from necrosed β cells. The saline-injected controls also received intraperitoneal glucose.

Serum glucose, total cholesterol, and lipoprotein levels were measured by the section of Laboratory Medicine at Chedoke-McMaster Hospitals, Hamilton, Ontario. Blood samples were taken 1 week after the alloxan injection and before the introduction of the egg yolk diet, after 2 weeks on the diet, and when the animals were killed. Serum low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglyceride levels were determined from the blood taken at the time of death.

Four groups of animals were examined. Group 1 (8 rabbits) was diabetic (serum glucose >10 mmol/L) and hyperlipemic (serum cholesterol >4 mmol/L); group 2 (5 rabbits) was normoglycemic and hyperlipemic; group 3 (4 rabbits) was diabetic and normolipemic; and group 4 (4 rabbits) was normoglycemic and normolipemic.

Group 1 animals were injected with alloxan, and 1 week later the animals that had developed hyperglycemia were fed the egg yolk-supplemented diet. Group 2 animals received a saline injection in place of the alloxan and were fed the egg yolk-supplemented diet. Group 3 animals were age-matched diabetic animals that continued to receive standard laboratory chow. Group 4 animals were age-matched but received no other treatment.

All animals were killed by an overdose of sodium pentobarbital 4 weeks after introduction of the egg yolk diet or after the same time on standard chow, ie, a total of 5 weeks after the alloxan injection.

Tissue Preparation

Tissues from every animal were sampled and processed in the same manner. Immediately after death the aortic arch and thoracic aortas were removed and placed in ice-cold tris(hydroxymethyl)aminomethane-buffered saline (TBS) and dissected free of adventitial connective tissue and fat. En face Hautchen preparations were made from full-circumference segments of the thoracic aorta between the second and fourth intercostal arteries, excluding the vessel orifices. Frozen sections were prepared from full-circumference segments of the aortic arch and thoracic aorta. The remaining aorta was opened and examined for the presence of macroscopic raised lesions.

Immunolabeling of Endothelial Cells for En Face Hautchen Preparations

The methodology is based on that described for use with fixed tissue. The immunostaining was performed by using mAbs to E-selectin and VCAM-1 on unfixed, unopened 1-cm segments of thoracic aorta before removal of the endothelial cells as Hautchen preparations. The procedures were performed at room temperature and were initiated as quickly as possible after removal of the tissue from the animal. The aortic segments were placed into 10-mL scintillation vials containing 0.5 mL protein blocking agent (AS/AP Immunostaining Kit; Bio/Can Scientific) for 10 minutes and then transferred to fresh scintillation vials containing the primary antibody for 1 to 2 hours. The tissue was washed three times in TBS (5 minutes each time) to ensure that all of the unbound primary antibody was removed.

<table>
<thead>
<tr>
<th>Table 1. Serum Glucose and Lipid Levels at the Time of Death</th>
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<tr>
<td>Group 1 (Diabetic Hyperlipemic)</td>
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<tr>
<td>Serum glucose, mmol/L</td>
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<tr>
<td>Rabbit 405</td>
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<td>Serum cholesterol, mmol/L</td>
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<td>Rabbit 405</td>
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<tr>
<td>Triglyceride, mmol/L</td>
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<td>Rabbit 405</td>
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<tr>
<td>LDL, mmol/L</td>
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<td>HDL, mmol/L</td>
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LDL indicates low-density lipoprotein; HDL, high-density lipoprotein; and BD, below detection. Values are mean±SD.
FIG 1. Photomicrographs showing vascular cell adhesion molecule–1 expression in Häutchen preparations from thoracic aortas of rabbits from each experimental group. The immunohistochemical marker, an alkaline-phosphatase–linked end product, appears dark brown, and the preparations are counterstained with hematoxylin. The areas illustrated are remote from vessel orifices. a, From a normoglycemic hyperlipemic rabbit (group 2). There are six adjacent stained cells (original magnification ×250). b, From a diabetic normolipemic rabbit (group 3). There is a focal accumulation of 10 stained cells (original magnification ×250). c, A lesion area from a diabetic hyperlipemic rabbit (group 1). The endothelial cells at the margin of the lesion are stained (original magnification ×150). d, A Häutchen preparation exposed to mouse anti-cytomegalovirus (isotype-matched, supernatant-derived, IgG,) and the same immunohistochemical detection procedure used with the specific monoclonal antibodies. The preparation includes approximately 300 adjacent endothelial cells and was taken from a diabetic normolipemic rabbit. There is no nonspecific immune staining (original magnification ×150).

The antibody was then added to the tissue for 1 to 2 hours. The antibodies to VCAM-1 and E-selectin were used in separate but adjacent segments of thoracic aorta.

To evaluate whether there was any nonspecific reaction to the IgGs used above, immunohistochemical staining was also performed using nonimmune mouse serum, nonspecific IgG, isotype-matched mAbs, or anti-cytomegalovirus (DAKO-CMV, CCH2, Dimension Labs Inc) in place of anti-VCAM-1 and anti–hepatitis B surface antigen (MAS 105, Dimension Labs Inc) in place of anti-E-selectin. The irrelevant antibodies were used at the same concentrations as the specific mAbs.

Häutchen Preparations

Immediately after the immunostaining the aortic segments were removed from the TBS, opened longitudinally, and cut into four pieces, each approximately 5×5 mm. The endothelial cells were removed from each piece of aorta on cellulose acetate paper; the portion of paper containing the cells was cut out and placed in a Petri dish with the endothelium facing upward. The chromogenic substrate solution, with the addition of levamisole to inhibit endogenous alkaline phosphatase, was applied to the endothelial cells on the cellulose acetate paper for 25 to 30 minutes, after which the color reaction was
stopped by placing the endothelial cells on cellulose acetate paper in TBS. The endothelial nuclei were stained with hematoxylin, and the preparation was rinsed in tap water, mounted in a 30% glycerol/distilled water mixture, and immediately photographed.

**Preparation of Frozen Sections**

Full-circumference 1-mm segments were snap-frozen on copper supports in liquid nitrogen with no other fixation. The frozen tissue was embedded in OCT (Tissue Tek, Miles Ltd). Five-micron sections were prepared by using a cryomicrotome and mounted on glass slides precoated with 3-aminopropyltriethoxysilane (Sigma Chemical Co). Serial sections were prepared from at least two blocks. Adjacent sections were stained with anti-vWF (to confirm the presence of endothelium), anti-VCAM-1, anti-E-selectin, anti-RAM-11 (anti-rabbit macrophage), and oil red O to indicate the presence of lipid. The immunostaining of the sections was performed according to the protocol used for the Häutchen preparations. Control preparations for the mAbs were performed using nonimmune murine IgG or the irrelevant mAbs and nonimmune goat IgG for the anti-vWF. During the staining procedures the slides were maintained at room temperature in a humidity chamber.
TABLE 2. Frequency of Endothelial Cell Expression of VCAM-1 or E-Selectin

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>VCAM-1</th>
<th>E-Selectin</th>
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<tr>
<td>Group 1, diabetic hyperlipemic</td>
<td>253±60 (7)</td>
<td>42±10 (7)</td>
</tr>
<tr>
<td>Rabbit 405</td>
<td>1053</td>
<td>28</td>
</tr>
<tr>
<td>Group 2, normoglycemic hyperlipemic</td>
<td>51±6 (5)</td>
<td>7±1 (5)</td>
</tr>
<tr>
<td>Group 3, diabetic normolipemic</td>
<td>117±2 (4)</td>
<td>17±5 (4)</td>
</tr>
<tr>
<td>Group 4, normoglycemic normolipemic</td>
<td>25±4 (4)</td>
<td>4±1 (4)</td>
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VCAM indicates vascular cell adhesion molecule. See "Methods" for definitions of groups. For each group, VCAM-1 versus E-selectin .05>P>.01.

Results

All the animals, including the alloxan-treated rabbits, appeared healthy, and there was no significant difference in body weights among the four groups of rabbits during the 5 weeks of the experiment.

Plasma Glucose, Cholesterol, and Lipoproteins

The mean serum glucose, total cholesterol, LDL, HDL, and triglyceride levels at the time of death are summarized in Table 1. The plasma glucose of all alloxan-treated diabetic animals (groups 1 and 3) was elevated at 1 week and remained at a constant level throughout the experiment. The total cholesterol levels for 7 of the 8 diabetic hyperlipemic animals (group 1) were similar to those of the normoglycemic hyperlipemic rabbits (group 2) at all times examined. The remaining diabetic hyperlipemic rabbit (rabbit 405) showed abnormally high total cholesterol throughout the experiment and a high triglyceride level at the time of death. It developed severe lipid-containing lesions and was excluded from the overall evaluation but is described separately below.

Lesion Development

All vessels were examined for the presence of raised, lipid-containing lesions. No macroscopic lesions were observed in the aorta of any animal except rabbit 405. Lipid-containing areas were identified by oil red O stain in cross sections of the aortic arch from 3 of 7 diabetic hyperlipemic animals (group 1) and 3 of 5 normoglycemic hyperlipemic animals (group 2). Lesions were observed in the Häutchen preparations of the thoracic aortas from 2 of 7 diabetic hyperlipemic (group 1) and 1 of 5 normoglycemic hyperlipemic (group 2) rabbits. Rabbits in group 3 (hyperglycemic and normolipemic) and group 4 (normoglycemic and normolipemic) did not develop lipid-containing lesions.

Incidence of VCAM-1 and E-Selectin in Häutchen Preparations

Almost all the endothelial cells were removed as a monolayer from each piece of the full-circumference segment of thoracic aorta used for the en face Häutchen preparations. The preparations stained with nonimmune mouse serum or type-matched IgG showed no evidence of nonspecific color reaction (Figs 1d and 2d). The cells that expressed VCAM-1 or E-selectin were clearly visualized by the dark brown end product of the immunohistochemical staining. Representative photomicrographs are presented in Figs 1, 2, and 5, and the frequency of expression (number of labeled endothelial cells per 10,000 cells) is summarized in Table 2.

Endothelial cells expressing either VCAM-1 or E-selectin were identified in the thoracic aortas of all animals, including the normoglycemic normolipemic age-matched control rabbits (group 4) (VCAM-1, 25±4 and E-selectin, 4±1 endothelial cells/10,000, respectively). In all treatment groups, cells expressing VCAM-1 were consistently more frequent (sixfold to eightfold) than those expressing E-selectin. The VCAM-1–or E-selectin–positive endothelial cells were distributed in a random fashion over the surface of the thoracic aorta except in the aortas of the hyperlipemic animals, in which large groups of stained cells were observed that were considered to be associated with lesion areas because of the arrangement of the endothelial cells (Figs 1, 2, and 5). VCAM-1 expression was frequently observed in groups of several adjacent endothelial cells (Fig 1), whereas E-selectin–expressing cells were generally seen as either single cells or groups of 2 or 3 cells (Fig 2). In endothelium taken from a lesion site, VCAM-1–expressing cells were distributed around and over the lesion but were largely absent from cells in the center, which were hexagonal rather than spindle shaped (Fig 1c). In similar lesions, endothelial cells that expressed E-selectin were distributed randomly over the lesion surface and especially at the extreme edges (Fig 2c).

Hyperlipemia (group 2) or hyperglycemia (group 3) alone significantly increased adhesion molecule expression, the effect of hyperglycemia being more pronounced (group 2: VCAM-1, 51±8 and E-selectin, 7±1 cells/10,000; group 3: VCAM-1, 117±2 and E-selectin, 17±5 bolts).
FIG 3. Photomicrographs showing serial sections from the aortic arch of a diabetic hyperlipemic (group 1) rabbit at the site of the intimal thickening associated with the orifice of the common carotid artery. Anti-von Willebrand factor stain indicated that the endothelial cell layer was intact (not shown). In panel a the immunohistochemical marker is the avidin-biotin peroxidase complex and appears red; in panels b and c it is an alkaline-phosphatase-linked end product that appears dark brown. The nuclei are counterstained with hematoxylin. a, Anti-vascular cell adhesion molecule-1-positive endothelial cells. (This section is not counterstained.) b, Anti-E-selectin-positive endothelial cells. c, RAM-11-positive macrophages in the intima. d, Oil red O-stained lipid (arrowheads) associated with macrophages and cells that did not stain with RAM-11 antibody (a through d, original magnification ×250).

FIG 4. Photomicrographs of serial sections from the aortic arch of a normoglycemic hyperlipemic rabbit (group 2) taken from the same region as that illustrated in Fig 3. a, Anti-vascular cell adhesion molecule-1-positive endothelial cells identified by the red avidin-biotin peroxidase complex end product. b, Anti-E-selectin-positive cells. c, RAM-11-positive cells in the intima. The brown peroxidase end product was used in the sections in b and c. d, Oil red O-stained lipid in RAM-11-positive cells and in smooth muscle cells (a through d, original magnification ×250).

cells/10,000). Together, hyperglycemia and hyperlipemia (group 1) elicited the highest level of adhesion molecule expression (VCAM-1, 253±60 and E-selectin, 42±10 cells/10,000); statistical significance was seen between groups 1 and 2 but not between groups 1 and 3 (Table 3).

Frozen Sections

The distribution of VCAM-1 and E-selectin in the aortic arch and thoracic aorta was evaluated from sections of frozen tissue and correlated with the presence of RAM-11-positive macrophages and oil red
FIG 5. Photomicrographs showing Häutchen preparations (a and b) and cross sections (c and d) of lesions that developed in the aortic arch and thoracic aorta of the diabetic grossly hyperlipemic rabbit (rabbit 405). a and b, Häutchen preparations from lesions in the thoracic aorta. a, Anti–vascular cell adhesion molecule-1 (VCAM-1)–stained cells surround the raised lesion and some cells in the center (arrowhead) that are neither spindle shaped nor stained by the antibody. The staining is heavy and it is difficult to identify individual endothelial cells in this instance. b, Anti–E-selectin–stained cells associated with a similar raised lesion. There are fewer stained cells distributed over and around the lesion. c and d, Serial cross sections of a lesion in the thoracic aorta. Anti–von Willebrand factor stain indicated that the endothelial layer was intact (not shown). c, Anti–VCAM-1–positive stain is present in the endothelium, and there is extensive medial smooth muscle cell staining. The peroxidase-linked immunocytochemical marker, visualized with 3-amino-9-ethylcarbazole, appears red, and there is no counterstain. d, RAM-11–positive cells are present in the lesion and one is seen in the superficial media. The immunohistochemical stain is shown by the dark brown end product. Lipid indicated by oil red O stain was confined to the region containing the RAM-11–positive cells (not shown) (a through d, original magnification X150).

O–stained lipid. The presence of endothelium was confirmed by staining with anti-vWF. Nonspecific staining was not seen using nonimmune serum or nonspecific isotype-matched antiserum in place of the mAbs. Sections of the aortic arch that included the intimal cushions formed by the thickening associated with the orifices of the brachiocephalic or subclavian arteries were examined in each animal. Representative photomicrographs are presented in Figs 3 and 4.

Groups 1 and 2: Diabetic Hyperlipemic and Normoglycemic Hyperlipemic

The accumulation of lipid and distribution of adhesion molecules in sections were similar in both groups of
hyperlipemic rabbit aortas. VCAM-1–positive endothelial cells were observed associated with oil red O–positive staining and with the intimal cushion at vessel orifices in the aortic arch of all animals. E-selectin was seen in single cells close to the areas in which VCAM-1 expression was present. Some of the lipid was located in the same region as RAM-11–positive cells, which formed a streak of lipid-containing macrophages (Figs 3 and 4). In other areas lipid was present where no RAM-11 stain was observed. This lipid may be in smooth muscle cells. In the diabetic hyperlipemic but not in the normoglycemic hyperlipemic rabbit aortas VCAM-1–positive endothelial cells were present over regions that did not contain RAM-11–positive cells or oil red O-stained lipid.

**Group 3: Diabetic Normolipemic**

There were no intimal lipid-containing lesions. In the sections of aortic arch both VCAM-1–positive cells and, very occasionally, E-selectin–positive cells were observed associated with an intimal cushion. In the remainder of the aortic arch and in the thoracic aortas occasional VCAM-1–positive cells were observed, but E-selectin–positive cells were not seen.

**Group 4: Normoglycemic Normolipemic**

Aortic sections of normoglycemic and normolipemic rabbit aortas did not show any morphological abnormalities such as lipid accumulation. Occasional VCAM-1–positive endothelial cells were associated with an intimal cushion in the aortic arch.

**Rabbit 405**

This diabetic hyperlipemic animal developed hyperlipidemia before the introduction of the egg yolk diet and had very high serum levels of total cholesterol (53.1 mmol/L) and triglyceride (63.1 mmol/L) at the time of death. The serum glucose level was similar to that of the other diabetic hyperlipemic or normolipemic rabbits (Table 1). The lipid-containing aortic lesions in this rabbit were more severe than in any other animal; the rate of expression of VCAM-1 was 1053 cells/10 000 and of E-selectin, 28/10 000 (Table 2). The cells associated with the lesions identified in the Häutchen preparations were very heavily stained for VCAM-1 and E-selectin (Fig 5a and 5b). In cross sections VCAM-1– and E-selectin–positive endothelial cells were observed in both aortic arch and thoracic aorta. In this animal there were raised lesions containing oil red O–positive, RAM-11–positive macrophages. The endothelium close to and over the lesions and the medial smooth muscle cells beneath the lesions were VCAM-1–positive (Fig 5c). E-selectin–stained endothelial cells were associated with the margins of lipid-containing lesions (Fig 5d).

**Discussion**

The frequency of endothelial cells expressing the leukocyte adhesion molecules VCAM-1 and E-selectin in rabbit aorta in vivo was quantitated in en face endothelial cell preparations. Alloxan-induced diabetes appeared to potentiate expression of both VCAM-1 and E-selectin, especially in a hyperlipemic setting. In all conditions, the frequency of VCAM-1–expressing endothelial cells was sixfold to eightfold higher than for E-selectin. This may reflect a longer duration of VCAM-1 expression and/or the type of stimuli responsible for induction.

The mechanism whereby adhesion molecule expression is induced in vivo remains to be determined. It is likely that cytokines are involved,13,14,27 and there is evidence that atherogenic lipids may also induce their expression,28 but hyperglycemia has not been identified as such a stimulus. From in vitro studies, both VCAM-1 and E-selectin are induced in endothelium by interleukin (IL)-1β,13,14 and tumor necrosis factor (TNF),27 IL-4 induces the expression of VCAM-1 but not E-selectin or ICAM-1.8 IL-1 or TNF induces E-selectin expression by 2 hours; expression peaks at 4 to 6 hours, and then declines rapidly.13 VCAM-1 is induced more slowly, reaches peak expression at 12 to 24 hours, and then declines. IL-1 and TNF typically are macrophage derived,29 although they can be produced by a variety of cells; notably in the arterial wall, endothelial and smooth muscle cells can produce IL-1,48 and IL-4 is a T-lymphocyte product. Monocyte adhesion to cultured endothelial cells in vitro is promoted by LDL,28 and β-very-low-density lipoprotein.31 More recently, minimally oxidized LDL, which is considered to play a key role in atherogenesis, has also been shown to increase monocyte but not neutrophil adhesion to endothelium in vitro.33 It has recently been demonstrated that VCAM-1 is induced in cultured arterial endothelial cells by lyso phosphatidylcholine, a component of atherogenic lipoproteins.28

Hyperglycemia appeared to be an effective stimulus to adhesion molecule expression in vivo in rabbits, and in patients with insulin-dependent diabetes increased circulating levels of ICAM-1 have been described.24 The mechanism for the induction of these adhesion molecules is not known, but hyperglycemia either directly or indirectly, as a result of nonenzymic glycation of proteins associated with endothelial cells and/or cytokines, may be involved. High levels of TNF-α are reported in the sera of newly diagnosed type 1 diabetic patients.35 The accumulation of white blood cells and the morphological alterations seen in the alloxan diabetic rabbit aorta may be a consequence of circulating cytokines, which induce endothelial injury and the expression of adhesion molecules in the absence of hyperlipemia. IL-1 and TNF may also be derived from macrophages, which are associated with the endothelium. This raises the possibility of an amplification mechanism and is consistent with the present observations that VCAM-1 was frequently seen in groups of adjacent endothelial cells. Moreover, both VCAM-1 and E-selectin were always present in endothelium overlying intimal RAM-11–positive macrophages, which did not contain lipid, as well as in the endothelium adjacent to the macrophage-rich fatty streaks. VCAM-1 was, however, also expressed in diabetic aortas in the apparent absence of any intimal macrophage. Thus, circulating cytokines and/or macrophage cells that can produce the inductive stimuli locally appear to be involved. It is likely that the expression of endothelial adhesion molecules may be influenced by the location within the vessel, because adhesion molecule expression and lipid accumulation were commonly observed in cross sections of the vessel at sites of branch vessels. The en face samples for quantification in this study were all taken from the same anatomic position in the aorta, and branch vessel orifices were avoided.

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In hyperlipemic rabbits, either diabetic or normoglycemic, cross sections of the vessel wall over foam cell lesions showed VCAM-1 expression in endothelial cells and in smooth muscle cells within the thickened intima. These smooth muscle cells were closely associated with RAM-11-positive macrophages. The endothelial expression of VCAM-1 has been described in hyperlipemic rabbits, and smooth muscle expression of VCAM-1 has been observed in cholesterol-fed rabbits and in human coronary arteries. The stimulus for the smooth muscle cells to express adhesion molecules may be derived from intimal macrophages.

In the en face preparations from hyperlipemic normoglycemic rabbit aortas, VCAM-1- and E-selectin-expressing endothelial cells were present in greater numbers than in similar preparations from normal rabbits. When lesion areas were included in the en face endothelial preparations, irrespective of the plasma glucose level, there was an increase in the rate of expression of both VCAM-1 and E-selectin in the endothelial cells associated with the lesions. These were observed particularly at the periphery of the lesions, a site where mononuclear leukocyte adherence and recruitment to lesions are most pronounced. These observations are consistent with VCAM-1 and E-selectin involvement in the recruitment of monocytes into the intima. It is tempting to speculate from the results in rabbit 405 that VCAM-1 rather than E-selectin plays the major role in monocyte recruitment, but these observations from one animal should be interpreted with caution.

In diabetic normolipemic rabbits at 5 weeks after alloxan treatment, endothelial adhesion molecule expression was even more frequent than in the normoglycemic hyperlipemic group, yet accumulations of intimal lipid were not found. The expression of the adhesion molecules is consistent with observations by scanning electron microscopy in an earlier study of increased adherence of leukocytes to the aortic endothelium in diabetic rabbits at 6 months of hyperglycemia and of occasional intimal macrophages in sections of the vessel wall. It is possible that endothelial cell expression of adhesion molecules in the alloxan-treated rabbit model of diabetes at 5 weeks is sufficient to support leukocyte adherence in arteries; however, for formation of intimal macrophage-rich, lipid-containing lesions, hyperlipemia may be necessary. The minimal potentiation of lipid deposition seen in the other diabetic hyperlipemic rabbits in the present study may reflect the short duration of the experiment, which was chosen to avoid the paradoxical response that an abnormal nonatherogenic pattern of lipoproteins develops in diabetic rabbits fed high levels of cholesterol for longer periods of time. This response pattern limits the use of this animal model in long-term studies of diabetes-related vascular disease.

En face Hautchen preparations stained immunohistochemically permitted detection of VCAM-1 and E-selectin expression in the normal rabbit aortic endothelium since more endothelial cells could be observed than in cross sections. Using random cross sections, we were not able to detect E-selectin expression in normal aortas. Hyperlipemia increased E-selectin expression only twofold, to 7 cells/10,000. Although predominantly localized over foam cell lesions, this extent of expression was low, consistent with the data we obtained from cross sections of aorta and with observations by others of advanced human atheromas. E-selectin is associated with the interaction of mononuclear cells and neutrophils with endothelium, but neutrophils were not observed associated with the arterial endothelium of diabetic rabbits. The low frequency (4 cells/10,000 expressing E-selectin and 25 cells/10,000 expressing VCAM-1) and the random spatial distribution of labeled endothelial cells bring into question the functional significance of the expression of E-selectin in the normal animal. The sixfold difference in the frequency of expression is similar to that in the stimulated animals and is consistent with the longer duration of VCAM-1 expression. The expression of adhesion molecules in normal animals and the rapid increase in expression after the induction of diabetes, even in a normolipemic setting, may be an indication that this is a response by endothelial cells to stimulation by a variety of agonists. In normal animals the adhesion molecules may be a consequence of ongoing or normally occurring damage or stimulation of endothelial cells, consistent with other observations of the failure to exclude dyes and the uptake of IgG by endothelial cells in normal rabbit aortas. The expression of these adhesion molecules in a hyperglycemic setting in the absence of hyperlipemia or lesion formation is consistent with chronic endothelial activation or injury associated with diabetes. However, the increase in adhesion molecule expression associated with hyperglycemia is indicative of an endothelial response to stimulation that can promote leukocyte infiltration into the intima and in a hyperlipemic setting and facilitate foam cell formation. Thus, the endothelial alterations reported in this study may provide some part of the explanation for the enhanced atherosclerosis associated with diabetes mellitus in humans.


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Increased expression in vivo of VCAM-1 and E-selectin by the aortic endothelium of normolipemic and hyperlipemic diabetic rabbits.
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