Intimal Alterations in Rabbit Aortas During the First 6 Months of Alloxan-Induced Diabetes

Steven Hadcock, Mary Richardson, Peter D. Winocour, and Mark W.C. Hatton

Diabetes mellitus is a major risk factor for atherosclerosis. Since endothelial alteration is probably associated with the development of atherosclerosis, we questioned whether morphological evidence of endothelial injury could be observed during the first 6 months of diabetes induced by a single intravenous injection of alloxan in normally fed rabbits compared with age-matched controls. Diabetes (plasma glucose >16 mM) was established by 5 days after alloxan injection. Endothelial alterations consistent with injury, including adhesion of white blood cells, platelets, and fibrin-like material to the endothelial surface, were seen in diabetic rabbit aortas by 2 weeks. These alterations became more severe during the next 6 months. Increased endothelial replication in diabetic vessels was shown by the uptake of tritium-labeled thymidine at 2 weeks and at 3 and 6 months. Hyperplasia of intimal smooth muscle cells progressed during 3 months after treatment. About one third of the diabetic rabbits also showed an elevated plasma cholesterol level, which correlated with increased intimal proliferation but not with endothelial injury or replication. The onset of alloxan-induced diabetes in rabbits is associated with nonnondening endothelial injury and subsequent intimal hypertrophy, changes that are consistent with atherogenesis. (Arteriosclerosis and Thrombosis 1991;11:517-529)

Onnondenuding injury to the arterial intima has been implicated in atherogenesis but has been demonstrated only in the initial stages of diet-induced atherosclerosis. The importance of mild injury as an atherogenic factor in a normal or even a mildly hyperlipemic setting remains to be defined.

Diabetes mellitus is a well-established risk factor for atherosclerotic vascular disease, which is prevalent in humans with both type I and type II diabetes. There are differences in the lipoprotein profiles associated with the two types of diabetes, but endothelial dysfunction, indicated by an increased loss of von Willebrand factor and decreased production of prostacyclin and plasminogen activator, has been described in both types of human diabetes as well as in experimental diabetes. It has been suggested that intimal injury is involved in diabetes-associated atherosclerosis, but any morphological evidence for endothelial injury associated with experimental diabetes is limited and mainly confined to reports of alterations at only one time interval after the induction of diabetes.

Experimental diabetes is readily induced in rabbits by administration of alloxan. Altered endothelial function is indicated by increased thromboxane A2 synthesis and decreased endothelium-dependent relaxation due to either increased thromboxane production or reduced synthesis of endothelium-derived relaxing factor. Alloxan-diabetic rabbits develop an alteration in low density lipoprotein (LDL) metabolism and a mild hypercholesterolemia, and when fed a diet that induces a mild hyperlipemia, they also develop more extensive atherosclerosis than do nondiabetic animals fed the same diet. A more severe atherogenic diet in diabetic rabbits is, paradoxically, associated with an abnormal very low density lipoprotein that is less atherogenic than that found in nondiabetic rabbits.

To determine if the development of diabetes is associated with morphological alterations in the arterial intima, we examined the aortas of rabbits at various intervals during the first 6 months of alloxan-induced diabetes mellitus and related these responses to the mild hypercholesterolemia that developed in some of these animals.
Methods

Animal Preparation

Eighty male New Zealand White rabbits were used in the study. These animals had an initial body weight of 3.5–3.8 kg and were maintained on standard laboratory chow with water ad libitum. Rabbits were selected at random and injected intravenously via a marginal ear vein with alloxan (65 mg/kg body wt) freshly dissolved in sterile saline (50 mg/ml). The remaining animals (controls) were injected with saline alone. All rabbits, including the saline-injected controls, received intraperitoneal glucose at 4 and 9 hours after the alloxan or saline injection to counteract the hypoglycemia caused by insulin release from necrosed β-cells due to the acute action of alloxan. Blood samples (in citrate) were taken for measurement of plasma glucose and cholesterol levels by the section of Laboratory Medicine at Chedoke–McMaster Hospitals (Hamilton, Canada). Plasma glucose levels were evaluated before the initial treatment and 24 hours later. Thereafter, plasma glucose levels were evaluated at weekly intervals and plasma cholesterol levels at biweekly intervals.

Morphological Examination

At each time interval of 1 day, 1 and 2 weeks, and 1, 3, and 6 months after the treatment with alloxan or with saline, rabbits were killed by perfusion fixation of the vascular system in situ. Three alloxan- and three saline-injected animals were killed at each time of 1 day, 1 week, and 1 and 6 months after alloxan or saline treatment. At 2 weeks and 3 months, three saline-injected controls and six alloxan-treated animals (three hypercholesterolemic and three normocholesterolemic) were selected for ultrastructural examination. At 2 weeks and at 3 and 6 months, four saline-injected controls and four hypercholesterolemic and four normocholesterolemic alloxan-treated animals were prepared for evaluation of the endothelial replication rate using tritium-labeled thymidine.

Perfusion fixation was performed while the rabbits were under general anesthesia (sodium pentobarbital, 35 mg/kg body wt). The right carotid and the left femoral arteries were isolated and cannulated with polyethylene tubing. The vascular system was perfused with Krebs–Henseleit solution (pH 7.35) at 100 mm Hg at room temperature for a fixed time (1 min/kg body wt). At this time, the perfusate was changed to 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.35), also at 100 mm Hg and room temperature. Pressure perfusion fixation was maintained for a minimum of 5 minutes. This protocol was strictly followed for all perfusions except for those animals used in the 3H-labeled thymidine uptake study, for which 4% paraformaldehyde was used as the primary fixative.

Preparation for Electron Microscopy

The descending aorta was removed intact from each animal and reimmersed in fixative solution for a further 6 hours at 4°C. One-centimeter-long, full-circumference samples from predetermined areas of the thoracic aorta (at the level of the third and seventh pair of intercostal arteries) and abdominal aorta (at the level of the left renal artery) were prepared for examination by scanning electron microscopy (SEM). Full-circumference samples adjacent to the SEM samples were prepared for examination by transmission electron microscopy (TEM); samples adjacent to these were frozen, and sections were cut and stained with Oil Red O for examination by light microscopy (LM). Samples for both TEM and SEM were postfixed in 1% aqueous OsO4 for 1 hour at 4°C and dehydrated through a graded series of ethanol. Samples for SEM were critical-point dried from CO2, sputter coated with gold, and examined with a Philips PSEM Model 501 (Philips, Eindhoven, The Netherlands). Samples for TEM were embedded in Spurr resin, and 1-μm sections were cut, mounted on glass slides, stained with Toluidine Blue, and examined by LM. Ultrathin sections for TEM were cut from areas identified in the LM sections as showing the greatest intimal thickening and examined with a Philips TEM Model 301.

Evaluation of Morphological Alterations

Evaluation of all morphological alterations was performed by two microscopists, both blinded to the origin of the tissues examined.

Intimal thickening. Intimal thickening was evaluated by LM from sections of the full circumference of the aorta. Briefly, two parameters were evaluated: 1) the proportion of the total circumference in which there was a visible separation of the endothelium from the internal elastic lamina. This was determined using a calibrated eyepiece and expressed as a percentage of the total circumference. 2) The number of layers of smooth muscle cells in the intima between the luminal surface of the internal elastic lamina and the luminal surface of the endothelium. This was counted from electron photomicrographs.

Endothelial ultrastructural alterations. Endothelial alteration was evaluated on the basis of specific features illustrated in Figures 1–7, which were observed by SEM and TEM photomicrographs. The following features were identified by SEM: adhesion of formed elements, white blood cells (WBCs) (Figure 1), platelets, red blood cells (RBCs), and fibrin (Figure 2); surface microvilli; bleb formation; and endothelial cell junction architecture (Figure 3). The following features were identified from TEM photomicrographs: endothelial cell junctional architecture and vacuolization of endothelial cell cytoplasm (Figure 4); endothelial and smooth muscle cell expression of metabolic organelles, including a dilated rough endoplasmic reticulum (Figures 4, 5, and 6); endothelial cell lysosomal content (Figure 6); and endothelial cell basement membrane thickening (Figure 7).
FIGURE 1. WBCs on the endothelial surface (e) of rabbit aorta 2 weeks after alloxan treatment. Upper left panel: Scanning electron (SEM) photomicrograph of one WBC; number of fine projections are evident in close contact with the endothelial surface. Upper right panel: SEM photomicrograph of area containing seven WBCs (one with arrowhead). On the surface of this vessel (e), average concentration is >50 WBCs/mm²; in this small area, concentration is >100 WBCs/mm². Lower panel: Transmission-electron (TEM) photomicrograph of WBCs on endothelial surface (e). One WBC and a portion of a smooth muscle cell (smc) are present in the subendothelial space. wbc, white blood cell; f, fibrin-like material.

Quantification

The number of WBCs on the luminal surface of the aorta (Figure 1) was quantified as previously described. Using an electronic dual-screen system (GW Electronics, Atlanta, Ga.), a defined area remote from vessel orifices was scanned systematically at instrumental magnification of ×640. The number of WBCs in each of 64 adjacent fields was counted and the total number expressed as WBCs per square millimeter. The data are presented in the text as mean, with the range given in parentheses.

The occurrence of the other features observed by SEM was quantified on a 1+ to 4+ basis, 1+ being least frequently and 4+ being most frequently expressed. The features identified by SEM were quantified by recording their frequencies in the same way as that used for WBCs. The features identified by TEM were quantified by noting the incidence of each feature in each endothelial cell in each section and expressing the incidence per 100 cells examined. At least two sections from each specimen were examined.

The data are presented as the mean±SD of the scores for each feature from all treated and age-matched control animals at each time interval.

Endothelial Replication

Endothelial replication was evaluated by quantifying the number of endothelial cell nuclei accumulating ³H-labeled thymidine. The endothelial cell monolayer was removed using a modification of the Hautchen technique previously described. ³H was localized by autoradiography. Briefly, samples were removed from the paraformaldehyde-fixed aortas, washed in distilled water for 20 minutes, and dehydrated through a graded series of ethanol.
dehydrated tissue was rinsed in acetonitrile and placed, endothelial side down, onto double-sided adhesive tape previously fixed to a glass slide. The tissue was immersed in 20% glycerol for 7-10 minutes and rapidly cooled on dry ice for 20-30 seconds; the media was then removed from the endothelium by gently stripping it with blunt forceps. The endothelial cells remaining on the slide were coated with
FIGURE 4. Upper panel: Transmission electron photomicrographs of endothelial cell showing vacuolated cell junction and large cytoplasmic vacuole. Lower panel: Endothelial cell with blebbed (r) and flat (f) junctions and dilated rough endoplasmic reticulum (arrowhead).

Kodak NTB2 (Eastman Kodak, Rochester, N.Y.) nuclear emulsion and developed after 2 weeks' exposure. The total number of endothelial cells and those with nuclei including silver grains were counted. The replication rate was expressed as labeled cells per 10,000 cells.

Statistical Analysis

The scores for the incidence of endothelial morphological features and the thymidine index were compared using a one-way analysis of variance (ANOVA) on untransformed data. The data for intimal thickening were compared using a one-way ANOVA after that data were subjected to variance-stabilizing transformations. The data derived from treated animals were compared with similar data from age-matched control animals. Significance is shown by $p<0.05$.

FIGURE 5. Transmission electron photomicrograph of endothelial cell with dilated metabolic organelles and an intimal smooth muscle cell containing a lipid droplet (I).

FIGURE 6. Transmission electron photomicrograph of endothelial cell containing lysosomes (ly) and dilated endoplasmic reticulum (arrowhead). Inset shows ribosomes associated with the material in the dilated cisternae of the endoplasmic reticulum.
Results

All the animals, including the diabetic animals, continued to gain weight and appeared healthy throughout the experiment.

Plasma Glucose and Cholesterol

The mean plasma glucose levels for the control and alloxan-injected rabbits throughout the experiment are summarized in Table 1. The plasma glucose levels of the alloxan-treated rabbits fell slightly during the first 24 hours, rose to a mean level of 17 mM by 1 week after treatment (control level, 6 mM), and remained close to this level throughout the rest of the experiment. The mean plasma cholesterol levels throughout the experiment are given in Table 2. The plasma cholesterol levels of approximately 60% of the alloxan-treated rabbits remained close to the mean control levels (0.7 mM). The remainder showed a small rise to 3.0 mM by 2 weeks, which was sustained for the 6 months’ duration of the experiment.

Morphological Observations

At all time intervals, the aortas of both control and test animals showed an intact endothelial layer with no evidence of frank endothelial desquamation. There was evidence of alteration in the aortic endothelium of alloxan-treated animals, which was consistent with injury.

A semiquantitative assessment of these alterations to the luminal surface of the aortas is summarized in Table 3 and the semiquantitative evaluation of alterations determined by TEM in Table 4. Examples of the specific features that were quantified are illustrated in Figures 1–7.

Saline-injected control animals. There was some injury observed in the aortas of control animals, but in all cases, this was significantly less than any alteration observed in the aortas of the age-matched diabetic animals.

In general, the endothelial surfaces of control aortas were mainly flat, and the majority of the intercellular junctional complexes were flat, with a small proportion being raised or blebbed. Small microvilli and large craters were occasionally seen in a few vessels. WBCs were observed at an average of <3/mm² (range, 2–3) at the beginning of the experiment, and by 6 months, the mean control value was 6/mm² (range, 5–9) and a very small number of discoid platelets were seen on the endothelial surfaces from the older control rabbits. Also, in older animals some evidence of fibrin-like material on the luminal surface was occasionally observed, and this was associated with the deposition of RBCs. In the aortic endothelial and smooth muscle cells of these animals, there was evidence of dilated metabolic organelles and occasional endothelial vacuoles and lysosomes.

Alloxan-injected diabetic animals. In the animals that were given intravenous alloxan and that became diabetic, there was evidence of endothelial alteration
or injury that progressed with time after the establishment of diabetes.

One day after injection. At this time, the blood glucose level was below normal values. The aortic endothelium of both control and test animals showed the formation of microvilli and occasional large crater at the endothelial surface. By SEM, the junctional architecture showed no unusual features, but by TEM, the junctions were vacuolized and there were cytoplasmic vacuoles.

One week after injection. Blood glucose levels had increased to 17 mM. The most marked alteration in the intima was the increase in the thickness of the endothelial cell basement membrane, which reached a value similar to that seen throughout the duration of the experiment; this was associated with the development of edema in the subendothelial space. There was an increase in the expression of a dilated rough endoplasmic reticulum in both endothelial and smooth muscle cells, and the endothelial cells from diabetic rabbits had more vacuoles than did the corresponding controls, but the appearance of the endothelial surface in SEM photomicrographs was essentially similar in both diabetic and control animals. The WBC accumulation on the endothelial surface was 5/mm² (range, 4–6) in the control vessels compared with 10/mm² (range, 9–12) in the alloxan-treated aortas. There was no evidence of smooth muscle cell migration or proliferation in the intima.

Two weeks after injection. Diabetic rabbits with raised plasma cholesterol levels were identified and compared with diabetic rabbits with plasma cholesterol levels close to control values. There were some marked alterations in the intima of the diabetic vessels, which showed little further increase in severity until 6 months after injury. By SEM, there were alterations in the endothelial cell junction and surface morphology, including an increased number of raised and blebbed junctions, microvilli formation, and both small and large blebs. These features were paralleled by the increase in lysosomes and in cell and junction vacuoles evident by TEM.

<table>
<thead>
<tr>
<th>Time after treatment</th>
<th>Control</th>
<th>Diabetic NC</th>
<th>Diabetic HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td>0.36±0.10 (n=4)</td>
<td>0.61±0.20 (n=4)</td>
<td>...</td>
</tr>
<tr>
<td>1 week</td>
<td>1.21±0.05 (n=4)</td>
<td>1.38±0.12 (n=4)</td>
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</tr>
<tr>
<td>2 weeks</td>
<td>0.78±0.08 (n=7)</td>
<td>0.93±0.27 (n=7)</td>
<td>3.31±1.51 (n=7)</td>
</tr>
<tr>
<td>1 month</td>
<td>0.89±0.10 (n=3)</td>
<td>1.06±0.28 (n=3)</td>
<td>...</td>
</tr>
<tr>
<td>3 months</td>
<td>0.63±0.24 (n=7)</td>
<td>0.72±0.09 (n=7)</td>
<td>2.04±0.14 (n=7)</td>
</tr>
<tr>
<td>6 months</td>
<td>0.59±0.07 (n=6)</td>
<td>0.86±0.09 (n=6)</td>
<td>...</td>
</tr>
</tbody>
</table>

Values are mean±SEM and are in millimoles per liter. NC, normocholesterolemic rabbits; HC, diabetic hypercholesterolemic rabbits.

**TABLE 3. Morphological Features Identified In Aortas of Alloxan-Treated Rabbits and Age-Matched Controls by Scanning Electron Microscopy**

<table>
<thead>
<tr>
<th>Feature</th>
<th>Denudation</th>
<th>Formed elements</th>
<th>Junctions</th>
<th>Cell surface</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>D</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td></td>
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<td>D</td>
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<td>D</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>D</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>WBCs/mm²</td>
<td>&lt;2</td>
<td>&lt;3</td>
<td>5±1.4</td>
<td>10±2.1†</td>
</tr>
<tr>
<td>Fibrin</td>
<td>0.1±0.03</td>
<td>0.1±0.03</td>
<td>0.2±0.1</td>
<td>1.1±1.1</td>
</tr>
<tr>
<td>Platelets</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.2±0.2</td>
</tr>
<tr>
<td>RBCs</td>
<td>0.3±0.5</td>
<td>1.0±0.5</td>
<td>0.8±0.1</td>
<td>1.0±0.3</td>
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<tr>
<td>Junctions</td>
<td>Flat</td>
<td>3.0±1.0</td>
<td>3.1±0.7</td>
<td>2.5±0.7</td>
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<tr>
<td></td>
<td>Raised</td>
<td>0.2±0.3</td>
<td>0.4±0.4</td>
<td>0.3±0.3</td>
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<tr>
<td></td>
<td>Blebbed</td>
<td>0.1±0.8</td>
<td>0.2±0.4</td>
<td>1.1±0.4</td>
</tr>
<tr>
<td>Cell</td>
<td>Flat</td>
<td>2.0±0.8</td>
<td>2.0±0.8</td>
<td>2.0±0.8</td>
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<tr>
<td></td>
<td>Microvilli</td>
<td>0.2+0.2</td>
<td>0.2±0.3</td>
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<tr>
<td></td>
<td>Large blebs</td>
<td>0.1±0.8</td>
<td>0.2±0.3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Small blebs</td>
<td>-</td>
<td>1.0+0.4</td>
<td>0.3±0.3</td>
</tr>
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</table>

*Mean±SD of counts. Remaining data are mean±SD of score for each feature based on a 1+ to 4+ basis, which reflects frequency of occurrence; -, not observed; 1+, least and 4+, highest frequency. All data are derived from three control animals and three to six diabetic animals.

Significant difference from age-matched controls is shown by †p<0.05; ‡p<0.01; §p<0.001.

C, control; D, diabetic; WBCs, white blood cells; RBCs, red blood cells.
The number of WBCs associated with the endothelium was 25/mm² (range, 2–51) and was increased compared with that of controls, which was 10/mm² (range, 2–12). In one aorta, which had more than 50 WBCs/mm², mononuclear cells were present in the subendothelial space (Figure 1). Occasional platelets were observed on the endothelial surface, together with more fibrin-like material and RBCs than in control animals. The expression of dilated rough endoplasmic reticulum was increased in both endothelial and smooth muscle cells in diabetic aortas. The thymidine index was increased in the diabetic animals. Compared with controls, the subendothelial edema increased, and this is reflected in the increased proportion of vessels showing intimal thickening. Smooth muscle cells were observed in the intimal space. There was no consistent difference in the extent of endothelial alterations in the aortas of rabbits that showed an elevated plasma cholesterol level compared with those with plasma cholesterol levels close to control values.

One and 3 months after injection. The alterations in the test animals seen at 2 weeks remained evident and were seen at approximately the same frequency and severity but were not associated with plasma cholesterol levels at 3 months after treatment. The number of adherent WBCs on test vessels was increased (mean, 15/mm² [range, 11–20] at 3 months) compared with control aortas (mean, 6/mm² [range, 3–9] at 3 months), and platelets that had some degree of shape change were seen on the endothelial surfaces of all the diabetic aortas, usually in the same location as adherent RBCs and usually associated with morphological evidence of fibrin deposition. These elements were often close to vessel orifices.

Six months after injection. By 6 months, there was an increase in the extent and severity of the expression of the alterations observed as at earlier time intervals.

There was no evidence of thrombus formation, but there was an increase in the amount of fibrin-like material and associated RBCs on the endothelial surface and in the numbers of adherent WBCs (mean, 32/mm² [range, 5–61] in treated aortas). There was a pronounced increase in the numbers of small and large endothelial surface blebs (Figure 3), and this was consistent with large numbers of cytoplasmic vacuoles and lysosomes. Fewer endothelial cell junctions were flat, many showed marked bleb formation, and by TEM, there was an increase in open and vacuolated junctions. The expression of metabolic organelles, including a dilated rough endoplasmic reticulum, was evident in both endothelial and smooth muscle cells.

Intimal Thickening

The data are presented in Table 5, and representative micrographs are presented in Figure 8. There is a progressive increase in the numbers of intimal smooth muscle cells and in the deposition of connective tissue in all vessels during the 6 months of the experiment. Intimal thickening was more marked in diabetic aortas than in the aortas of nondiabetic age-matched animals, and it continued to progress for the first 3 months after treatment with no further thickening at 6 months. The proportion of the circumference of the aorta that showed intimal thickening was significantly increased in all diabetic aortas by 2 weeks after treatment. In the hypercholesterolemic but not in the normocholesterolemic animals examined at 2 weeks and 3 months after treatment, the number of

### Table 4. Morphological Features Identified in Aortas of Alloxan-Treated Rabbits and Age-Matched Controls by Transmission Electron Microscopy

<table>
<thead>
<tr>
<th>Feature</th>
<th>1 day</th>
<th>1 week</th>
<th>2 weeks</th>
<th>1 month</th>
<th>3 months</th>
<th>6 months</th>
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<tr>
<td></td>
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<td>D</td>
<td>C</td>
<td>D</td>
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<td>D</td>
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<tr>
<td><strong>Endothelial cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilated RER</td>
<td>13±5</td>
<td>12±2</td>
<td>20±5</td>
<td>32±8</td>
<td>23±3</td>
<td>45±6†</td>
</tr>
<tr>
<td>Vacuolation</td>
<td>21±3</td>
<td>19±5</td>
<td>37±3</td>
<td>54±10</td>
<td>37±3</td>
<td>64±6‡</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>22±0</td>
<td>22±0</td>
<td>38±2</td>
<td>51±13</td>
<td>41±1</td>
<td>60±9§</td>
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<td></td>
<td></td>
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<tr>
<td>Vacuolated</td>
<td>11±1</td>
<td>9±2</td>
<td>21±1</td>
<td>34±4†</td>
<td>15±2</td>
<td>39±4§</td>
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<tr>
<td>Intimal SMCs</td>
<td></td>
<td></td>
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<tr>
<td>Dilated RER</td>
<td>11±1</td>
<td>11±4</td>
<td>20±0</td>
<td>33±4†</td>
<td>21±3</td>
<td>44±7§</td>
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<td><strong>Subendothelial space</strong></td>
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<tr>
<td>Basement membrane</td>
<td>12±2</td>
<td>11±3</td>
<td>18±1</td>
<td>41±15†</td>
<td>17±1</td>
<td>43±8§</td>
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<tr>
<td>Edema</td>
<td>9±2</td>
<td>9±2</td>
<td>13±3</td>
<td>30±12†</td>
<td>14±4</td>
<td>28±8†</td>
</tr>
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</table>

Values are mean±SD of counts of incidence of feature per 100 cells. All data are derived from three control animals and three to six diabetic animals.

C, control; D, diabetic; RER, rough endoplasmic reticulum; SMCs, smooth muscle cells.

Significant difference from age-matched controls is shown by †0.05>p>0.01; §0.01>p>0.001; ¶p<0.001.
intimal smooth muscle cells was significantly greater than in the age-matched controls.

**Endothelial Cell Uptake of [3H]Thymidine**

The data are presented in Table 6. Endothelial cells in mitosis were noted in diabetic vessels at all time intervals. The number of endothelial cells that demonstrated uptake of [3H]thymidine was 0.9±0.3/10,000 cells in control aortas, and this increased to 4.3±1.5/10,000 cells in diabetic aortas at 2 weeks after injection with alloxan. At 3 months after injection, the labeling was 1.0±0.4/10,000 cells in the control compared with 1.8±0.4/10,000 cells in the diabetic rabbit aortas. Six months after treatment, 1.6±0.2/10,000 cells were labeled, compared with 0.9±0.1/10,000 in age-matched controls. The increase in labeling index in the aortas of treated animals compared with age-matched controls was significant at each time interval. There was no significant difference between the labeling index in the aortas of diabetic rabbits with raised or normal levels of plasma cholesterol at any of the time intervals examined.

**Lipid Accumulation**

No section stained by Oil Red O showed evidence of lipid accumulation. Small lipid droplets were seen in the myointimal cells of diabetic aortas but not in age-matched controls at 3 and 6 months (Figure 5).

**Discussion**

These studies demonstrate that at 2 weeks after alloxan induction of diabetes in rabbits, there was morphological evidence of arterial endothelial injury, increased endothelial replication, and intimal hyperplasia, but not lipid accumulation. During the subsequent 6 months, the degree of intimal injury gradually increased, and this increase paralleled but was more severe than alterations in age-matched control animals. The intimal hyperplasia did not progress past 3 months but, up to that time, was most marked in those diabetic animals that also showed a mild hypercholesterolemia.

We observed no evidence of endothelial cell denudation, but there were alterations in the ultrastructure of diabetic aortic endothelial cells that are consistent with injury. De-endothelialization had previously been reported in alloxan-treated rabbit aortas, but this was possibly due to the silver-staining procedures used during tissue preparation. However, the injury indicated by the increased argyrophilia is consistent with the endothelial alterations reported here. Since the vessels examined in the present study were prepared strictly according to the same protocol, which has been shown previously to minimize nonspecific alterations in endothelial morphology, it is likely that the changes we observed are due to subtle endothelial injury that is related directly to the diabetic state. These alterations result in increased endothelial cell turnover and therefore...
endothelial cell desquamation, but not denudation of the subendothelium.

Most of the changes in endothelial cell morphology observed in the aortas of diabetic rabbits, including formation of blebs and craters, altered or raised cell margins, and microvillus formation, were similar to those described in other situations of endothelial stimulation and are features associated with a number of noxious stimuli such as endotoxin, serum sickness, and reperfusion after ischemia. The formation of small blebs seen increasingly in the diabetic vessels at all time intervals after 2 weeks was similar to that described in endothelial cells in culture exposed to diabetic serum. The blebs evident by SEM were seen in TEM as cytoplasmic and membranous vacuoles (Figure 3), and these accumulated in parallel with an increased number of lysosomes. Increased numbers of lysosomes have been associated with atherogenesis, and the features listed above are considered to be consistent with endothelial injury although no clear explanation for the cause or consequence of these structures has been given.
Adhesion to the endothelial surface by WBCs, RBCs, and platelets was observed regularly in the aortas of diabetic rabbits. The WBCs were quantified over a large area of the aortic endothelial surface, and it should be noted that the distribution was not uniform, but the cells tended to concentrate in focal areas (Figure 1). In section, the WBCs were mononuclear cells, and these cells were occasionally present in the intima but were never observed to contain lipid. Increased accumulation of WBCs has been described as an early response to hyperlipemia, but in the diabetic aortas, the number of WBCs on the endothelial surface was independent of the plasma cholesterol level. It is possible that the accumulation of WBCs is a result of endothelial stimulation, resulting in the synthesis of chemotactic factors such as interleukin-1 or the expression of endothelial-leukocyte adhesion molecules. The increased expression of a dilated endothelial rough endoplasmic reticulum has been described in situations such as inflammation and is associated with increased metabolic activity and with increased adhesion of WBCs. von Willebrand factor has been localized in the endoplasmic reticulum of rat aortic endothelial cells after endotoxin- or balloon-induced injury. It is possible that an alteration in the production of von Willebrand factor is part of the response to endothelial injury and is consistent with other alterations observed.

Platelets were present on the endothelial surface in all the diabetic animals examined after 3 months and were seen in some animals at earlier time intervals, indicating an alteration of the endothelial cells in the diabetic state. The endothelial cells to which platelets were attached appeared intact by SEM, and the platelets were associated with the cell surface, not primarily the cell junction region. It is generally considered that platelets do not adhere to normal endothelium in vivo, although platelet binding to cultured endothelial cells stimulated with thrombin or injured or virally transformed endothelium has been reported. The presence of platelets usually coincided with increased numbers of RBCs and evidence of fibrin-like material on the endothelial surface. The adhesion of platelets to the endothelium may be a result of increased fibrinogen binding by the platelets, especially as the platelets observed on the diabetic endothelium showed some evidence of activation as indicated by a change in shape. Fibrin has been observed in the aortas of diabetic rats, and fibrinogen uptake and accumulation have been shown to be increased in the diabetic compared with the nondiabetic rabbit aorta. An increased adhesion of RBCs to the endothelial surface was observed by SEM, and this is consistent with observations using cultured endothelial cells from diabetic humans, which show increased adhesion to RBCs.

The increase in the thickness of the endothelial basement membrane is well documented in the microvascular circulation in diabetes when it appears to develop over a period of years. In the present study, the aortic endothelial basement membrane appeared thickened by 1 week after treatment (Figure 7) and was close to maximum by this time. This increase occurred simultaneously with subendothelial edema and the increase in the proportion of the vessel wall showing intimal thickening. It is possible that this is related to an increase in endothelial permeability associated with cell damage, including alterations in junctional morphology (Figure 4). Arterial basement membrane thickening may involve a different mechanism than in the microcirculation.

The animals used in this study were approximately 6–9 months old (3.5 kg body weight) at the beginning of the experiment, and during the 6-month duration of the experiment, the aortas of control animals increasingly showed certain morphological features that have been associated with stimulation or a response to injury in younger rabbits (2.5 kg and approximately 3 months old). These were consistently much less severe than those observed in diabetic animals and were considered to be associated with the normal aging process. Changes seen in the endothelium of both test and control animals 1 day after the injection of alloxan may have been caused by the stress of the procedures, especially the intraperitoneal injections of glucose. It is also possible that alloxan may exert a nonspecific toxic effect on the endothelium. However, alloxan has a half-life of approximately 5 minutes in solution, and the endothelial alterations were observed in parallel to the rise in plasma glucose levels and persisted for at least 6 months.

The increased uptake of H-labeled thymidine by endothelial cells in diabetic aortas is consistent with endothelial cell proliferation. This may reflect replacement of endothelial cells after an increased cell death rate, or it may indicate an increase in the total number of cells associated with growth. In these studies, the animals were mature, and the increase in thymidine index was evaluated against age-matched controls. It is most likely that the increase in endothelial cell replication rate is a consequence of the injury induced by the hyperglycemia as indicated by the alterations in morphology. The increased rate of cell replication was not dependent on the plasma cholesterol levels seen in the present study, whereas an increase in endothelial replication rate was observed in nondiabetic rabbits with serum cholesterol levels of approximately 300 mg/dl after 20 weeks of diet. It is possible that the replication rate in diabetic vessels may be further stimulated if the plasma cholesterol level is increased by diet-induced hypercholesterolemia, but in this study the increased endothelial cell replication rate is related to the diabetics. Since diabetic serum will stimulate the growth of cultured fibroblasts, smooth muscle cells, and endothelial cells, it is possible that the stimulation of endothelial cell proliferation is a consequence of an insulin-like growth factor.
Intimal hyperplasia appeared to increase with the early progression of diabetes. Compared with age-matched controls that show progressive intimal thickening with increasing age, there is a further increase in the proportion of the intima that is thickened. The numbers of smooth muscle cells in the region between the internal elastic lamina and the endothelium is increased in diabetic animals and is significant in hypercholesterolemic diabetic rabbits. The smooth muscle cells in the intima of the diabetic rabbit aortas showed more evidence of dilated metabolic organelles than did those in the age-matched control vessels. This is consistent with these cells' being stimulated to express the secretory phenotype, which is capable of migration and proliferation. This may be due to a variety of stimuli, including growth factors of endothelial cell or platelet origin derived from the WBCs that are present on the endothelial surface and diabetes-related insulin-like growth factors. The precise role for cholesterol in this response is unclear.

Alloxan-induced diabetes results in non-necroendothelial injury to rabbit aortic endothelium, and this may contribute to the increased risk for atherosclerosis seen in diabetes. These observations provide support for the concept that atherogenesis is initiated by endothelial injury.

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