Immunohistochemical Detection of Macrophages and T Lymphocytes in Atherosclerotic Lesions of Cholesterol-Fed Rabbits

Göran K. Hansson, Paul S. Seifert, Gun Olsson, and Göran Bondjers

Human atherosclerotic plaques contain significant numbers of T lymphocytes and monocyte-derived macrophages. Cytokines released from activated T lymphocytes induce aberrant expression of major histocompatibility complex class II (Ia) antigens by vascular smooth muscle cells and may also regulate cell proliferation and metabolism in the vessel wall. We have analyzed the arteries of cholesterol-fed rabbits to study the sequence of lymphocyte and monocyte entry into the forming atherosclerotic lesion. Rabbits were fed 0.3% cholesterol for 1–10 weeks, and monoclonal antibodies to rabbit leukocyte differentiation antigens and Ia antigen were applied to sections of the aorta. Monocytes were already observed 1 week after initiation of cholesterol feeding, and they accumulated in the intima, where they formed the bulk of the foam cell–rich lesion. T lymphocytes also adhered to the aortic surface from 1 week onward, and also accumulated in the lesion, although in lower proportions than did monocytes. In 10-week lesions, approximately 6% of cells expressed the T-lymphocyte marker L11/135. Ia antigen expression was frequent throughout the lesion in all phases of its development, and most of the Ia-expressing cells could be identified as monocyte-derived macrophages. These data indicate that the cholesterol-fed rabbit is a useful model for studying the role of monocytes and T lymphocytes in atherosclerosis. (Arteriosclerosis and Thrombosis 1991;11:745–750)

The atherosclerotic plaque bears many similarities to chronic inflammatory lesions. One of the hallmarks of inflammation is the activation of the immune system. This is reflected in the infiltration of lymphocytes and monocytes and the expression of class II major histocompatibility complex (MHC) antigens in the inflammatory lesion. We and others have recently found that these phenomena also take place in atherosclerosis. T lymphocytes as well as monocytes are present in the human atherosclerotic plaque, and class II MHC antigens are expressed by many cells of the lesion, both macrophages and smooth muscle cells. However, it has not been possible to deduce the time sequence of these inflammatory phenomena from analyses of human surgical or autopsy material. We have therefore turned to an animal experimental model to investigate the temporal relation between class II MHC expression, lymphocyte and monocyte infiltration, and development of the atherosclerotic lesion.

In rabbits, addition of moderate amounts of cholesterol to the diet results in rapid development of arterial lesions. In the early stages of lesion formation, occasional foam cells are present in the subendothelial intimal space. The lesions progress into fatty streaks and may with time even develop a fibrous component of smooth muscle cells and extracellular matrix components, thus resembling the human atherosclerotic plaque.

We have studied the inflammatory and immunologic components of atherosclerosis in the diet-induced rabbit model. We present evidence for adhesion of monocytes and T lymphocytes to the arterial intima at an early stage of hypercholesterolemia. Expression of Ia antigen is high from the start of...
Lesion formation. T lymphocytes are present, and monocytes/macrophages dominate the fatty streak-type lesions in these rabbits.

**Methods**

**Animals**

Four-month-old rabbits of the New Zealand White strain were obtained from Lidköpings kaninfarm, Lidköping, Sweden. They were fed standard rabbit chow supplemented with 0.3% cholesterol, and serum cholesterol was analyzed monthly by a standard enzymatic procedure (Monotest Cholesterol, Boehringer Ingelheim, Ingelheim, F.R.G.). Groups of four rabbits were killed after 1, 3, 6, and 10 weeks on the diet. They were sedated with diazepam, anesthetized with halothane, and fixed by perfusion with 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at a pressure of 100 cm H₂O. Samples of the descending part of the thoracic aorta were snap frozen in liquid N₂ as described.

**Antibodies**

Monoclonal antibodies against cell type-specific rabbit antigens were used for immunohistochemical analysis. They are listed in Table 1. The L11/135 hybridoma was obtained from the American Type Culture Collection (Rockville, Md.) and grown in Dulbecco's modified Eagle's medium (GIBCO, Paisley, Scotland) supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 µg/ml streptomycin, nonessential amino acids, glutamine, oxaloacetic acid, and insulin as described. Hybridoma cells were grown in the mass culture system described by Sjögren-Jansson and Jeansson, and immunoglobulins were purified from the supernatant by protein A-Sepharose chromatography (Bio-Rad MAPS II, Richmond, Calif.). All other antibodies were obtained from the producer either as supernatants or as purified immunoglobulin (see Table 1). All antibodies were used at optimal dilutions determined by checkerboard titrations on sections of rabbit spleen and aorta. Staining of such sections was also performed to verify the cellular specificity of each antibody.

**Immunohistochemistry**

Staining was performed essentially as described, except that alkaline phosphatase instead of peroxidase was used for immunoenzyme localization. In brief, 10-µm cryostat sections were fixed in ethanol, incubated with monoclonal antibodies followed by species-specific biotinylated F(ab')₂ fragments of anti-mouse immunoglobulin G (IgG) (Amersham, Amersham, U.K.) and alkaline phosphatase-conjugated avidin (Dakopatts, Copenhagen, Denmark). Antibody binding was visualized with an alkaline phosphatase substrate kit (AP substrate kit I, Vector, Burlingame, Calif.). Controls included antibodies to irrelevant antigens (species-specific anti-human leukocyte differentiation antigens) as well as omission of primary antibody. The percentage of cells positive with a certain antibody was determined by counting 100 cells in a segment from the surface to the internal elastic lamina at the site of maximal lesion thickness. Corresponding fields were chosen in serial sections.

**Results**

Rabbits were fed a diet supplemented with 0.3% cholesterol for varying time periods ranging from 1 to 10 weeks. The changes in serum cholesterol levels with time on diet are shown in Figure 1. After 10 weeks on the diet, the average serum cholesterol concentration was 17.69±6.61 mmol/l (mean±SD), corresponding to 682±255 mg/dl. Small intimal foam cell accumulations could be observed after 2–3 weeks on the cholesterol-rich diet, and macroscopically detectable lesions were found in rabbits treated with cholesterol for 6 and 10 weeks.

We used a battery of cell type-specific monoclonal antibodies to detect different hematopoietic cells in these lesions (Table 1). RAM11 binds to a protein that is expressed on monocyte-derived macrophages in rabbits. It has recently been used to successfully identify such cells in atherosclerotic lesions. Antibody 1.24 recognizes the majority of rabbit leukocytes and appears to be analogous to the leukocyte common antigen in humans. L11/135 is a pan-T lymphocyte marker, which recognizes T lymphocytes in blood and tissues but does not cross-react with other leukocytes or any other cell type. The cytotoxic/suppressor subset of rabbit T lymphocytes is detected by the CD8 equivalent, 12.C7. Finally, the 2C4 antibody binds to the rabbit homologue of the class II MHC antigen.

Occasional RAM11⁺ monocytes and L11/135⁺ T lymphocytes were seen at the endothelial surface after 1–3 weeks of cholesterol feeding (Figure 2). After 6 weeks on the diet, small clusters of cells were observed in the intima (Figure 3). The majority of them were always RAM11⁺ monocyte-derived macrophages (Figure 3, upper panel, Table 2). Smaller amounts of L11/135⁺ T lymphocytes were also invariably present in these lesions (Figure 3, lower panel, Table 2). Approximately two thirds of the macrophages expressed Ia, as judged from serial sections.
stained with RAM11 and 2C4 (Figure 3, middle panel, Table 2). Occasional spindle-shaped cells that were presumably intimal smooth muscle cells could also be detected in these early lesions.

The lesions in rabbits fed cholesterol for 10 weeks were of the fatty streak type. They were dominated by lipid-laden foam cells, most of which expressed Ia, and the macrophage marker RAM11 (Figure 4, Table 2). Expression of RAM11 was less intense in the lower part of the lesion than in the subendothelium, and the 1.24 epitope was only expressed by subendothelial macrophages, suggesting that there may be phenotypic differences between macrophages in different parts of the lesion.

Comparison of serial sections implied that most of the Ia+ cells were RAM11+ macrophages. In addition, there was a population of Ia+ spindle-shaped cells that were probably Ia+ smooth muscle cells.

L11/135+ T lymphocytes were also present in these lesions but to a lower extent (Figure 4, Table 2). The frequency of T cells varied between regions and was highest subendothelially and in the shoulder regions. Only a small proportion of the cells reacted with the 12.C7 monoclonal antibody, suggesting that very few CD8-type T lymphocytes were present in the lesions.

**Discussion**

Rabbits respond to cholesterol feeding by developing arterial intimal lesions that resemble human fatty streaks, that is, they largely consist of lipid-laden foam cells. With time, these lesions may acquire a fibrotic component consisting of smooth muscle cells and extracellular matrix components and transform into fibrous plaque-like lesions. Our present data confirm the previous observation that the foam cells that build up the early lesions are largely derived from blood monocytes.

The lesions of cholesterol-fed rabbits resemble human atherosclerotic lesions in that they both contain large amounts of monocyte-derived macrophages. In contrast, the lesions that form after mechanical arterial injury in rats are dominated by proliferating smooth muscle cells and contain very few hematopoietic cells. Human atherosclerotic lesions exhibit a significant inflammatory component, with large amounts of T lymphocytes as well as macrophages and with high frequency of Ia antigen expression. The latter phenomenon suggests that an immune response may be taking place, since Ia antigens (in humans, HLA-DR, -DQ, and -DP) are pivotal for the recognition of foreign antigens by T lymphocytes. Our recent observation that T lymphocytes in human plaques express activation markers such as the interleukin-2 receptor further supports the idea that an immune response may be taking place.

The similarities in macrophage accumulation between the human disease and the cholesterol-fed rabbit model suggested to us that the latter could be useful also for studies of inflammatory and immune aspects of atherosclerosis. We have therefore analyzed monocytes/macrophages, T lymphocytes, and Ia expression in

FIGURE 4. T lymphocytes (arrows) in fatty streak-like lesion of a rabbit fed cholesterol for 10 weeks. L111/135 monoclonal antibody, hematoxylin counterstain. ×80.

TABLE 2. Frequency of Different Cell Types Defined by Monoclonal Antibodies in Intima and Lesions at Various Stages

<table>
<thead>
<tr>
<th>Cell type</th>
<th>MAb</th>
<th>Time on cholesterol diet (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>L11/135</td>
<td>7.4±5.6</td>
</tr>
<tr>
<td>Ia+ cells</td>
<td>2C4</td>
<td>41±14</td>
</tr>
<tr>
<td>Macrophages</td>
<td>RAM11</td>
<td>73±13</td>
</tr>
</tbody>
</table>

Data represent percent antibody-positive cells in lesions of different age (mean±SD, n=4 per group).

MAb, monoclonal antibody.

the early phases of atherogenesis in cholesterol-fed rabbits. Immunohistochemical staining of serial cryostat sections was used to detect binding of cell type-specific monoclonal antibodies. The use of frozen rather than paraffin-embedded sections was necessary to avoid destruction of antigenic epitopes but inevitably resulted in thicker sections and suboptimal morphology. This, in turn, made it difficult to compare reactivity with different antibodies on the single-cell level. Instead, conclusions concerning macrophage Ia expression, for example, had to be based on comparison of similar fields and on frequencies of positive cells.
Adhesion of monocytes and T lymphocytes to the aortic surface was detectable already after 1 week of cholesterol feeding, and intimal lipid-laden monocyte-derived macrophages were detected at 3 weeks. Small lesions could be observed at 6 weeks, and by 10 weeks, large fatty streaks were present throughout the aorta.

Ia antigen, the major rabbit class II MHC antigen, was present on the majority of cells in the lesions at all time points studied. Ia staining was strong and uniform and could be detected intracellularly as well as on cell surfaces, suggesting a high synthesis of Ia protein. These observations indicate that Ia is expressed in experimental atherosclerosis from the earliest detectable stage and onwards.

The majority of Ia-expressing cells were macrophages that expressed the RAM11 antigen and, at a lower frequency, the 1.24 antigens. Ia antigens are expressed constitutively in this type and are upregulated by γ-interferon.

γ-interferon can induce de novo expression of class II MHC molecules in smooth muscle cells. Occasional Ia-expressing smooth muscle cells appeared to be present, but the frequency of these cells could not be determined since the smooth muscle contribution to the experimental lesions under study was very low.

The function of Ia molecules is to participate in antigen recognition. T lymphocytes are activated by a complex of foreign antigen and Ia molecule on the surface of the antigen-presenting cell. The high frequencies of Ia-expressing cells and T lymphocytes indicate that the potential for antigen presentation, and thus, for an immune response, is present in dietary-induced experimental atherosclerosis.

The use of the rabbit T cell–specific monoclonal antibody L11/135 permitted us to detect T lymphocytes in rabbit vascular tissue by immunohistochemistry. At early stages of hypercholesterolemia, T cells could be seen adhering to the endothelial surface before any fatty streaks had developed. In fully developed lesions, T cells constituted 5.7% of the cell population. Although this frequency is slightly lower than that of T cells in complicated human atherosclerotic plaques, it is possible that these cells may play a biologically significant role in the development of the rabbit lesions. Any further comparisons between the human plaques previously studied by us and others and the present rabbit model are obviously limited by the differences in age of lesions and species.

The findings of both T lymphocytes and Ia-expressing cells that may have the capacity to present antigens to cells raise the possibility that a specific immune response is taking place in cholesterol-induced atherosclerosis. It has been shown that modified lipoproteins such as glycosylated low density lipoprotein can induce immune responses, but there is no reason to assume that this particular response was taking place in the present model.

In addition to their role in antigen recognition, T lymphocytes are important producers of paracrine factors that can regulate the functions of surrounding cells. The activated T lymphocyte secretes γ-interferon, which can inhibit cell proliferation in vascular smooth muscle cells and endothelial cells and also activate macrophages. It also produces lymphotoxin, an analogue of tumor necrosis factor, and we recently found that this protein modulates γ-interferon–induced gene expression in smooth muscle cells.

Finally, it has recently been shown that type I interferon, which is produced by virally infected cells, can suppress lesion formation in cholesterol-fed rabbits. Although the secretory patterns and spectra of effects differ in many respects between the two types of interferons, they share some effects. It is therefore possible that the effect of parenterally administered type I interferon could, to some extent, mimic the effect of endogenous type II (γ) interferon.

In conclusion, the present study shows that Ia-expressing cells, largely of monocytic origin, and T lymphocytes are abundant in the aortic lesions of cholesterol-fed rabbits. Both cell types were present at all stages, initially adhering to the surface at prelesion stages and later on as major components of the fatty streak–like lesions. These observations support our hypothesis that cells of the immune system participate in the formation of atherosclerotic lesions. They open up the possibility to use the cholesterol-fed rabbit for studies of immunologic aspects of atherosclerosis.

Acknowledgments

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