Differential Accumulation of Intimal Monocyte-Macrophages Relative to Lipoproteins and Lipofuscin Corresponds to Hemodynamic Forces on Cardiac Valves in Mice

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We have studied the distribution of monocyte-macrophages, lipids, and lipoproteins in sections of aorta and aortic valves from mice fed an atherogenic diet. By immunocytochemical analysis with Mac-1 and F4/80 antibodies, apolipoprotein B antibody, and oil red O staining, three discrete regions were identified: 1) the aortic wall of the sinus of Valsalva, which contained deposits of lipid that colocalized with lipoproteins and monocyte-macrophages; 2) the sides of the aortic valve leaflets facing the ventricle, which did not contain lipids or lipoproteins but which were lined with macrophages that colocalized with lipofuscin; and 3) the sides of the leaflets facing the aorta, which did not contain lipids, lipoproteins, monocyte-macrophages, or lipofuscin deposits. This pattern of distribution resembles the expected distribution of mechanical forces, especially those of systolic blood flow, which in the three areas are predominantly 1) low-shear disturbed flow, 2) high-shear laminar flow, and 3) low-shear laminar flow, respectively. These findings suggest that lesions in the mouse closely resemble early atherosclerotic lesions in humans and other primates with respect to monocyte-macrophage and lipoprotein accumulation. The results also strikingly demonstrate that the accumulation of monocyte-macrophages and lipoproteins can occur independently, with spatial differences corresponding to the distribution of hemodynamic forces. (Arteriosclerosis and Thrombosis 1991;11:947–957)

Early atherosclerotic lesions are characterized by the accumulation of monocyte-macrophages and lipoproteins in the arterial intima. We and others are attempting to develop a mouse animal model to identify and characterize genetic factors contributing to the early inflammatory events of atherosclerosis. Studies from several laboratories have revealed that certain strains of mice, including C57BL/6J, when fed a high-fat diet (reviewed in Reference 3), develop early atherosclerotic lesions resembling fatty streaks in humans. As in human atherosclerosis, the development of lesions in the mouse is strongly influenced by the levels of plasma lipoproteins; in particular, low levels of high density lipoproteins cosegregate in several genetic crosses with lesion development. As part of an effort to further examine the validity of the mouse model for studies of the early stages of atherosclerosis, we have performed immunohistochemical studies of the lesions by use of specific antisera to apolipoprotein (apo) B, a major protein of low density and very low density lipoproteins, and to monocyte-macrophages. The results indicate that both apo B and monocyte-macrophages colocalize with lipid in the aortic sinus of Valsalva, a region of high predilection to lesion development in the mouse.

During the course of these studies we unexpectedly observed the accumulation of monocyte-macrophages along with a dark pigment, identified in this article as lipofuscin, located on the ventricular sur-
Sinus of Valsalva

Outflow side

Inflow side

Outflow side

L. atrium

Aortic root

Aortic leaflet

Inflow side

L. ventricle

Diagnosis 1. Diagram of anatomy and flow patterns of the aortic valve. Vortices form in the sinuses of Valsalva during ejection. Flow separates beyond the tip of the valve and rejoins the aortic wall (reattachment), where the shear layer may become disturbed. L, left.

face of the aortic valves. As illustrated in Figure 1, this surface is directly exposed to outflow from the heart. In contrast to the aortic lesions, these areas were totally devoid of lipid and apo B-containing lipoproteins, and they occurred only on the ventricular surface and never on the aortic surface of the valve leaflets. These distinct patterns of lipoprotein and macrophage accumulation on the two surfaces of the aortic valves and on the aortic wall provide evidence for the role of fluid shear stress or other mechanical forces in early lesion development. Regions of the vascular tree most susceptible to atherosclerosis are typically sites of branch points or bends where hemodynamic and solid mechanical forces change rapidly with respect to time and position. The present results with the mouse model suggest that macrophages may accumulate independently of lipoprotein deposition, with a distribution reflecting the mechanical environment.

Methods

Materials

OCT embedding medium was purchased from Fisher Chemical Co., Santa Clara, Calif. Oil red O was from Sigma Chemical Co., St. Louis, Mo. Mac-1 antibody was from Boehringer Mannheim, Indianapolis, Ind. F4/80 antibody was from Bioproducts for Science, Indianapolis, Ind. The avidin-biotin peroxidase staining system (Vectastain, Elite ABC kit) was from Vector Laboratories, Burlingame, Calif. The peroxidase chromogen kit (AEC) was purchased from Biomedica Corp., Foster City, Calif.

Animals

C57BL/6J female mice, about 3 months of age at the beginning of the study (Jackson Laboratory, Bar Harbor, Me.), were fed an atherogenic diet based on the Thomas-Hartroft formula, together with standard mouse chow (Purina #5005) in a 1:3 ratio for 15–22 weeks. This diet contained 9% fat, 1.25% cholesterol, and 0.5% cholic acid. Animal procedures were in accordance with institutional guidelines.

Tissue Preparation

The mice were killed by cervical dislocation, and the heart was excised and placed in phosphate-buffered saline (PBS), pH 7.2, for 5–10 minutes. For evaluation of lesions and immunostaining the heart was embedded in OCT compound, frozen on dry ice, and held at −70°C until cryosectioned. The method of sectioning and lesion evaluation has been described previously. The basal portion of the heart and the proximal ascending aorta were sectioned transversely into 10-μm-thick slices. Serial sections were collected on polylysine-coated slides and stored at −70°C.

Immunocytochemistry

Consecutive 10-μm sections taken through the heart and aorta (in groups of three) were used to evaluate neutral lipid accumulation, presence of monocyte–macrophages, or presence of apo B. Lipoprotein accumulation was evaluated by use of a monospecific polyclonal antibody prepared in rabbit against rat apo B. Lipoprotein accumulation was evaluated by use of a monospecific polyclonal antibody prepared in rabbit against rat apo B. Immunoblotting and immunoprecipitation studies have demonstrated the specificity of the antibody, which cross reacts well against mouse apo B-100 and apo B-48. Slides were fixed in acetone for 15 minutes at room temperature for monocyte–macrophage immunostaining and in 4% paraformaldehyde in PBS for 5 minutes at 4°C for anti-apo B immunostaining. Both sets were pre-blocked for endogenous peroxidase by exposure to H2O2 and immunostained with the avidin–biotin immunoperoxidase system, followed by color production with the peroxidase chromogen 3-amino-9-ethylcarbazole and counterstained with hematoxylin. Slides were immunostained overnight at 4°C with rat monoclonal antibody to mouse Mac-1 antigen at 1:250 dilution, with rat monoclonal antibody to mouse macrophage (F4/80) at 1:1,000 dilution, or with affinity-purified rabbit anti-rat apo B antibody at 0.2 μg/ml. Control studies were performed with nonspecific rat and rabbit sera. Sections of liver and spleen were also excised, fixed, and immunostained for Mac-1 and F4/80 antigens as positive controls.

Histochemistry

Lipid deposits were identified by use of oil red O with hematoxylin and light green counterstain. To clarify the nature of the pigment deposits, specimens...
were rinsed in PBS, fixed in 4% buffered formaldehyde for 48 hours, dehydrated through a graded series of ethanol concentrations, and then embedded in paraffin.\textsuperscript{11} Serial 4-\(\mu\)m-thick sections were collected on consecutive gelatin-coated slides. The pigment was evaluated by use of a series of stains described by Bancroft and Stevens\textsuperscript{11}: 1) oil red O stain after overnight xylene extraction to test for xylene-insoluble neutral lipids; 2) the Ziehl-Neelsen method to test for acid-fast lipid; 3) periodic acid-Schiff staining to test for aldehydes; 4) Schmorl’s ferric ferricyanide as a general lipofuscin stain; and 5) Prussian blue iron stain to test for iron-containing pigment such as hemosiderin.

**Hemodynamic Analysis**

To assess the flow conditions at the aortic valve leaflets, we applied the data of Peacock\textsuperscript{12} to conditions in the mouse. To determine the Reynolds number, hemodynamic parameters for the mouse were estimated as follows: aortic diameter=1 mm; body weight=20 g by direct measurement; heart rate=600 beats/min; blood viscosity=0.04 poise; and blood density=1.06 g/ml from established values.\textsuperscript{13} Cross-sectional area of the open valve was estimated to equal cross-sectional area of the aorta, based on the assumption that the valve leaflets are parallel to the aortic wall during systole.\textsuperscript{13} The Womersley parameter for oscillatory flow was calculated from the equation \(\alpha = \pi (\omega / v)^{1/2}\), where \(\alpha\) is the Womersley parameter, \(r\) is aortic radius, \(\omega\) is the oscillatory frequency in radians per second, and \(v\) is kinematic viscosity. Vessel wall components were assumed to be rigid, and the blood was assumed to behave as a Newtonian fluid. To correct for the absence of valve flow during diastole, which constitutes approximately two thirds of the cardiac cycle, flow velocity during systole was calculated as three times the mean aortic flow divided by valvular cross-sectional area.

**Results**

**Monocyte–Macrophage and Apolipoprotein B Accumulation in Aortic Lesions**

Previous studies have shown that “susceptible” strains of mice such as C57BL/6J develop intimal thickening and lipidosis in the proximal regions of the ascending aorta in response to a high-cholesterol, high-fat diet.\textsuperscript{3,4,7,8} In C57BL/6J female mice maintained for 15 weeks on a high-fat, high-cholesterol diet,\textsuperscript{7} we observed lesions predominantly in the proximal 350 \(\mu\)m of the aorta. In agreement with the results of Paigen and colleagues,\textsuperscript{8} we found that lesions were more likely to occur in aortic sections closer to the heart than in sections closer to the aortic arch. Also in agreement with previous work,\textsuperscript{4} the total cholesterol levels of C57BL/6J mice fed chow and high-fat diets were 76±9 and 224±52 mg/dl (mean±SD), respectively. The high density lipoprotein cholesterol levels of the mice fed chow and high-fat diets were 48±1 and 30±6 mg/dl, respectively. In an effort to further examine the nature of the lesions in this animal model, we used immunocytochemical methods to evaluate lipoprotein and monocyte–macrophage accumulation, which is typically observed in early atherosclerotic lesions in primates and various other mammalian models for the disease.

Monocyte–macrophage accumulation was studied with the use of two rat monoclonal antibodies, Mac-1\textsuperscript{14} and F4/80,\textsuperscript{15} which bind to antigens on mouse monocytes–macrophages. Mac-1 recognizes antigens present on neutrophils as well as monocytes–macrophages, whereas F4/80 is highly specific for mouse monocytes–macrophages. In all of our experiments Mac-1 and F4/80 staining colocalized on tissue sections (data not shown). In control experiments preimmune sera and the omission of second antibodies were used to confirm the specificity of the staining with the three antibodies (data not shown).
The aortic wall of the sinus of Valsalva had lipid-staining regions (Figures 2 and 3, upper panel) and varying numbers of macrophage-derived foam cells, consistent with the aspect of early fatty streaks (Figure 3, middle panel). These deposits also stained for antibody to apo B (Figure 3, lower panel) and were closely associated with monocyte–macrophages identified by the Mac-1 antigen (Figure 3, middle panel).
Definite staining for apo B was also found in association with Mac-1 antibody in the adventitia (Figure 4, upper and lower panels) in the absence of neutral lipid staining.

Mac-1-positive cells were more densely stained at the shoulders (Figure 5, upper panel) than at the centers of lesions (Figure 5, middle panel). The macrophages in the central portions of the lesions appeared to be more distended (Figure 5, middle panel), presumably as a result of lipid accumulation and foam cell formation (Figure 5, lower panel), raising the possibility that the reduced staining intensity resulted from a simple "dilution" of the antigen. Mac-1-reactive cells were confirmed to be monocytes rather than granulocytes or null cells by staining with F4/80, a rat monoclonal antibody with high specificity toward mouse macrophages, which colocalized with Mac-1-positive regions (data not shown).

Focal lipid deposits identified by oil red O staining were present in the thoracic and abdominal aortas of the mouse after 15 and 22 weeks of a high-fat diet (Figure 6). These areas of focal lipid insudation were small and apparently not very advanced. Moreover, macrophages could not be detected in the lesions with the use of Mac-1 immunostaining (data not shown). It is noteworthy that we failed to observe significant lipid deposition at the ostia of the intercostal arteries, an area with a high predilection to lesion development in certain other mammals.

Monocyte-Macrophage and Lipopfuscin Accumulation in the Absence of Lipoprotein Accumulation in the Ventricular Face of Aortic Valves

In contrast to the lipoprotein and lipid accumulation observed in the aortic sinus, we failed to observe any evidence of such accumulation on either the inflow or the outflow side of the aortic leaflets (Figures 2 and 7, upper left panel). However, during the course of these studies we noted the presence of striking pigment deposits in the subendothelium of the ventricular surface of the aortic valve (Figure 7), despite the absence of foam cells, oil red O staining, and anti-apo B staining. As discussed below, the ventricular aortic valve surface is an area of high
flow. The pigments were found to occur in close association with monocytes, as indicated by Mac-1 staining (Figure 7, upper right and both lower panels). These Mac-1-reactive cells were also confirmed to be monocytes rather than granulocytes or null cells by staining with F4/80 (data not shown). The pigment was present on unstained sections (data not shown) and was limited to the ventricular (high-flow) face of...
the valve leaflet (Figure 7, both upper panels and lower left panel). Thus, in contrast to the ventricular face no pigment deposits, macrophages, lipoproteins, or lipids were seen on the aortic face of the leaflets. Mac-1 staining was also found in association with pigment on the atrial face of the anterior mitral leaflet. The presence of monocytes in association with pigment was not observed in other areas of the heart and aorta or in other tissue such as liver and spleen.

Histochemical analysis of the pigment was most consistent with that for lipofuscin/ceroid. Prussian blue reaction for iron was negative, indicating the absence of hemosiderin. After xylene extraction, which solubilizes lipids not highly cross linked or complexed to proteins, oil red O stain was faintly positive at the site of pigmentation, together with some annular structures. Periodic acid–Schiff staining was positive in the areas surrounding the pigment, but it did not change the dark color of the pigment. Ziehl-Neelsen staining for acid-fast material was positive/negative. Reaction with Schmorl’s ferric ferricyanide gave the pigment deposits a navy blue hue, consistent with that of lipofuscin. Histochemical results are expected to vary, depending on the degree of oxidation of the lipofuscin. The more dense, presumably older, and more oxidized pigments tended to obscure histochemical colorations, as described by Bancroft and Stevens.11

While the macrophage- and lipoprotein-containing lesions on the aortic wall are observed only when susceptible mice are challenged for prolonged periods with a high-cholesterol, high-fat diet, the lipofuscin deposits are frequently observed in mice maintained on chow diets. Nevertheless, there appear to be genetic factors affecting lipofuscin deposition in the aortic valves. Thus, of 26 C57BL/6J mice examined after 15 weeks of a high-fat diet, all except two contained lipofuscin deposits. On the other hand, mice of the BALB/cJ strain (of the same sex and age and maintained under identical conditions) only infrequently (one of 11 examined) contained lipofuscin deposits. It is interesting to note that the accumulation of lipofuscin is correlated with the development of lesions in the aortic wall; thus, C57BL/6J mice are susceptible to both, whereas BALB/cJ mice are relatively resistant to both. Whether the lipofuscin deposition and aortic lesion development are controlled by common genetic factors is unknown.

Hemodynamics
As shown in Figure 1, the aortic root was subdivided into three regions differing in intimal accumulation of lipid, lipoproteins, and monocyte–macrophages: the aortic wall of the sinus of Valsalva, the aortic side of the aortic leaflet, and the ventricular side of the aortic leaflet (Figure 8). Using an estimate of cardiac output from empirical equations,13 we calculated the mean systolic Reynolds number at the mouse aortic valve to be approximately 35, with a mean systolic velocity of 12.8 cm/sec and a kinematic viscosity of 0.037 poise. Under steady flow conditions, this value for the Reynolds number is well below the threshold for turbulence in the mainstream flow. To correct for oscillatory flow, the dimensionless Womersley parameter was calculated to be 2.0. Based on the analysis by Peacock,12 these results predict that laminar vortexes form within the sinus of Valsalva, including the outflow side of the aortic leaflet, and that transitional (disturbed) flow occurs at the reattachment zone on the aortic wall of the sinus. Thus, as a generalization the ventricular surface is exposed to high-shear laminar flow, the aortic wall of the sinus is exposed to low-shear disturbed flow, and the aortic face of the valve leaflet is exposed to low-shear laminar flow.

Discussion
The goal of the present study was to determine whether lesions in atherosclerotic mice fed an atherogenic diet demonstrate the same inflammatory
Pigment deposits on aortic valve leaflet. Upper left panel: Low-power photomicrograph showing oil red O staining on the aortic wall of sinus but not on the leaflet. Upper right panel: Photomicrograph of consecutive section showing anti-Mac-1 antibody staining of both sinus and leaflet in association with pigment. Lower left panel: High-power photomicrograph of anti-Mac-1 stained section of aortic valve showing alignment of dark brown pigment along the flow-exposed surface of the leaflet in association with monocytes. Lower right panel: High-power photomicrograph showing anti-Mac-1 staining in intimate contact with the pigment. V, ventricular aspect; A, aortic aspect; S, sinus of Valsalva.

Upper left panel, ×225; lower left panel, ×360; lower right panel, ×1,440.
features as do early atherosclerotic lesions in primates and certain animal models. Our results reveal that mice fed an atherogenic diet exhibit inflammatory monocyte-macrophage infiltration and lipoprotein accumulation in the intima of the aortic wall of the sinus of Valsalva similar to that observed in the early atherosclerotic lesions of primates. We also observed that macrophage but not lipid or lipoprotein accumulation occurs on the inflow surfaces of the aortic valve leaflets. These results indicate that under certain conditions, two of the early events in atherosclerosis, that is, lipoprotein accumulation and inflammatory monocyte–macrophages, do not always coincide. In addition, we observed deposits of pigment, most likely consisting of lipofuscin/ceroid, lining the subendothelial space of the inflow surfaces of the aortic valve leaflets and the atrial surfaces of the mitral valve leaflets; these deposits were closely associated with the Mac-1 antigen but not with neutral lipids or foam cells.

Pathology

The mouse has been suggested to be an animal model useful for the identification and characterization of genetic factors contributing to the early stages of atherosclerosis. Previous studies have shown a variety of phenotypes among various inbred strains of mice fed an atherogenic diet. However, detailed characterization of the cellular components have not yet been reported. The present results have provided additional information about these early events occurring in the mouse aorta with exposure to a high-fat diet.

In accordance with previous observations in primates, neutral lipid accumulation was accompanied by apo B and monocyte–macrophage accumulation. Mac-1 staining of monocyte–macrophages appeared very dense at the shoulders of the lesions due to the presence of numerous cells that were not yet lipid filled. The staining became more diffuse in the central portions of the lesions where the monocytes appeared distended and engorged with lipid, thereby possibly decreasing the density of the cell surface Mac-1 antigen. This is in accordance with the results of Rosenfeld and Ross, who have shown that the shoulders of the lesions are areas of monocyte infiltration and proliferation and that focal regions are areas of lesion growth.

As in other animal models, the atherosclerotic lesions of the mouse appear to progress distally along the aorta with continued feeding of the atherogenic diet. We have noted oil red O staining in the subendothelium of the thoracic, abdominal, and iliac regions of the aortic wall after 15–22 weeks of the high-fat diet. However, at these times these regions are devoid of Mac-1 staining, indicating the absence of monocyte–macrophages. These areas may represent very early events leading to the formation of fatty streaks. Schwenke and Carew have reported that the initiation of atherosclerotic lesions is associated with increased concentrations of low density lipoprotein in the artery wall, consistent with the concept that lipid accumulation is a necessary first step in the pathogenesis of the fatty streak.

Hemodynamic Forces

The reasons for segregation of lipid, macrophages, and pigment deposition to different areas of the valve associated with different fluid and solid mechanical forces are not clear. Flow patterns are particularly complex at the aortic valve and differ markedly on either side of the valve: in general, there is high-shear laminar flow on the ventricular face (inflow) and low-shear disturbed flow on the aortic face (outflow) where vortices or eddies form. Many secondary components may occur as well. When the aortic valve opens, vortices develop behind the leaflets in the sinuses of Valsalva (Figure 1). The leaflets lie parallel to flow in the mainstream, which is generally laminar, being balanced by forces on the two sides. Flow separates beyond the tip of the valve and rejoins the aortic wall of the sinus of Valsalva (flow reattachment), where the shear layer may become disturbed (transitional) or completely turbulent. In addition to shear other mechanical forces act on the leaflet, including internal stress and pressure gradients.

Compared with the geometry used by Peacock, the mouse aortic valve is smaller, resulting in a lower Reynolds number, and heart rate is greater, resulting in a lower threshold for disturbance, based on results of oscillatory flow analysis by Nerem and Seed. This value of the Womersley parameter lowers the critical peak (threshold) Reynolds number to between 500 and 2,000. Due to its small dimensions, the Reynolds number for flow in the mouse aortic valve remains well below this critical value.
Hemodynamic forces are known to affect structural and metabolic aspects of vascular endothelial cells. Lesions grossly resembling atherosclerosis have been described for human aortic and mitral valves. However, to our knowledge the presence of pigment deposits on the inflow (ventricular) side of the leaflets has not been previously described. High-shear forces on the leaflet may lead to increased cell damage or turnover, constituting "wear and tear" long associated with lipofuscin. Macrophages may then accumulate to clear cellular debris. Flow disturbances on the aortic wall of the sinus may increase lipoprotein retention and oxidation, with macrophages accumulating in response to the oxidized lipid. Low-shear laminar flow on the outflow surface of the valve presumably would not predispose to the accumulation of either lipoprotein or lipofuscin.

The aortic valve is also subject to solid mechanical stresses due to the stretching that occurs when it supports the weight and pressure of the column of blood in the ascending aorta during diastole. Because the leaflet curvature reverses between systole and diastole, bending stresses are alternately compressive and extensile on the two faces of the leaflets. Analysis by Thubrikar et al predicts higher stress on the ventricular face during diastole and higher stress on the aortic face during systole. Similarly, the pressure is greater in the aorta during diastole and in the ventricle during systole. Although the quantitative differences may explain the present histological findings, the relation to distribution of subendothelial deposits is less obvious than it is for flow patterns. Direct mechanical abrasion of the ventricular surfaces during valve closure is another potential contributing factor. However, such contact is limited to the thin "line of coaptation" at the leaflet tips; the subendothelial deposits of pigment are not.

**Pigment**

Our results indicate that the likely identity of the pigment observed on the outflow side of the aortic leaflet is lipofuscin/ceroid. Lipofuscin and ceroid are composed of mixed complexes of breakdown products from free radical–induced lipid oxidation of cellular debris, together with protein. The mechanism of formation of these complexes remains obscure. Lipofuscin is often described as a wear-and-tear pigment, suggesting association with physical forces leading to cell damage. Such forces probably occur on the flow-exposed surface of cardiac valves, where shear may approach 180 N/m, exceeding the level at which erythrocytes hemolyze and platelets are damaged. Shear is also associated with increased cell turnover, which could account for the presence of both lipofuscin and a macrophage response.

Ceroid has been described in advanced atherosclerotic plaques, where it is mostly extracellular, as well as in lipid-laden foam cells found in both fatty streaks and more advanced lesions. The association of ceroid with macrophages led to the hypothesis that it was formed as a result of oxidative mechanisms within the macrophage or from lipid oxidation occurring in the atherosclerotic intima. In vivo, ceroids exhibit a wide variety of forms and frequently undergoes progressive changes with time. Their accumulation in the leaflets may be the result of mechanical forces or inherent differences in the endothelium or subendothelium of the two surfaces. Mechanical forces may also enhance lipid oxidation by increasing energy requirements of endothelial cells. Due to the additional energy requirement of adhering to the matrix against strong time-varying shear forces, endothelial cell mitochondrial respiration may increase, leading to increased formation of oxygen radicals and increased oxidation of lipid elements.

Preliminary observations of these mice suggest that the degree of pigmentation increases but that the distribution remains the same with duration of the high-fat diet. We also observed a clear genetic difference in pigment accumulation between the susceptible C57BL/6J mice, which generally contained pigment, and the resistant BALB/cJ mice, which rarely contained pigment. It is interesting to speculate that the genetic factors affecting lesion development in the aortic wall may also predispose monocyte–macrophage and lipofuscin accumulation.

These studies suggest that the mouse may serve as a suitable model for study of the early events in atherogenesis such as lipoprotein retention and modification, monocyte recruitment and conversion to macrophages, and foam cell formation. Moreover, these studies further suggest a role of hemodynamic forces in determining the localization and characterization of the lesions that develop.

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References


KEY WORDS • atherosclerosis • monocyte-macrophages • cardiac valves • lipoproteins • hemorheology • lipofuscin/keroid
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