Inflammation in Atherosclerosis
Lesion Formation in LDL Receptor–Deficient Mice With Perforin and Lyst<sup>beige</sup> Mutations

Natalie K. Schiller, William A. Boisvert, Linda K. Curtiss

Objective—Natural killer (NK) cells have been identified in human vascular pathologies. In this study, we identified NK cells in aortic root atherosclerotic lesions of low density lipoprotein (LDL) receptor–deficient (LDL<sup>r–/–</sup>) mice. To characterize the role of NK cell–mediated cytolysis in atherosclerosis, we generated C57Bl/6 double-mutant mice by crossing LDL<sup>r–/–</sup> mice with NK cell–defective Lyst<sup>beige</sup> mice (creating beige,LDL<sup>r–/–</sup> mice) and with perforin-deficient mice (creating Pfp<sup>–/–</sup>,LDL<sup>r–/–</sup> mice).

Methods and Results—Male mice (8 to 10 weeks old) were fed a high-fat diet to induce atherosclerosis. Compared with LDL<sup>r–/–</sup> mice, beige,LDL<sup>r–/–</sup> mice had impaired NK cell cytolytic activity and significantly increased atherosclerosis (<i>P</i> < 0.05). Pfp<sup>–/–</sup>,LDL<sup>r–/–</sup> mice had impaired NK cell cytolytic activity, yet they had lesions that were similar to those of control mice. This suggested that NK cell cytolysis did not play a significant role in atherosclerosis and that the exacerbated atherosclerosis of the beige,LDL<sup>r–/–</sup> mouse was independent of impaired NK cell cytolytic activity. Therefore, we investigated the role of T and B lymphocytes in atherosclerosis of beige mice by crossing them with recombinase activator gene 1–deficient LDL<sup>r–/–</sup> mice (Rag1<sup>–/–</sup>,LDL<sup>r–/–</sup> mice), thus creating beige,Rag1<sup>–/–</sup>,LDL<sup>r–/–</sup> mice. As in the double-mutant study, beige,Rag1<sup>–/–</sup>,LDL<sup>r–/–</sup> mice had significantly increased lesions compared with Rag1<sup>–/–</sup>,LDL<sup>r–/–</sup> control mice.

Conclusions—Therefore, the Lyst<sup>beige</sup> mutation in LDL<sup>r–/–</sup> mice has proatherogenic properties that are independent of NK cell–mediated cytolysis and lymphocyte-mediated acquired immunity. (Arterioscler Thromb Vasc Biol. 2002;22: 1341-1346.)

Key Words: atherosclerosis ▪ perforin ▪ Chédiak-Higashi syndrome ▪ lysosome ▪ natural immunity

Atherosclerosis is a multifactorial disease characterized, in part, by the accumulation of lipids and inflammatory factors within the vessel wall. Increased adhesiveness and permeability of the endothelium leads to the accumulation of leukocytes, including monocyte-derived macrophages and T lymphocytes. These cells constitute early fatty streaks and remain present within lesions throughout more advanced stages of the disease.1–5 Other lymphocytes such as B cells and natural killer (NK) cells, although found more rarely than T lymphocytes and macrophages, have also been observed in atherosclerotic lesions.6–9

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NK cells are large granular lymphocytes that use cytoplasmic granules to kill certain tumor cells and virally infected cells. NK cells have been identified and implicated in other human vascular pathologies, such as abdominal aortic aneurisms and Takayasu’s arteritis.10–12 The present study was designed to delineate what role NK cell cytolysis plays in atherogenesis. We generated double-mutant mice that were prone to atherosclerosis and also had defects in NK cell cytolytic activity. One strain was generated by crossing LDL receptor–deficient (LDL<sup>r–/–</sup>) mice with perforin-deficient mice (creating Pfp<sup>–/–</sup>,LDL<sup>r–/–</sup> mice). Perforin, also known as pore-forming protein, is found in cytoplasmic granules of NK cells and cytotoxic T lymphocytes. On activation, NK cells and cytotoxic T lymphocytes release perforin, which assemblies to form a pore on the membrane of the target cell, including transformed or virally infected cells expressing altered class I major histocompatibility complex. This pore permits the entry of granzymes simultaneously released from the NK cell granules and leads to lysis of the target cell. Perforin deficiency results in defective granzyme-mediated cell killing, which in NK cells renders them unable to lyse the target cells.

We generated a second double-mutant strain by crossing LDL<sup>r–/–</sup> mice with natural mutant Lyst<sup>beige</sup> mice, creating beige,LDL<sup>r–/–</sup> mice, which are also defective in NK cell cytolysis. Lyst<sup>beige</sup> mice are the animal homologue of a rare
autosomal-recessive disorder in humans called Chédiak-Higashi syndrome (CHS). The mouse gene Lyst (or lysosome trafficking regulator) encodes for a protein that has been suggested to be involved in the exchange of membrane material between the trans-Golgi network and late endosomes. The hallmark of the CHS/beige mutation is the presence of huge perinuclear granules, resulting in increased rates of infection and an immune deficiency related primarily to defective NK cell cytosis.

Unexpectedly, compared with LDLr−/− control mice, the double-mutant beige, LDLr−/− and Pfp−/−, LDLr−/−, Pfp−/− mice, in which NK cell cytolytic activity was severely impaired, had disparate atherosclerosis. The beige, LDLr−/−; Pfp−/− mice had exacerbated atherosclerosis, whereas the Pfp−/−, LDLr−/− mice did not. To confirm these results as well as to identify the role of acquired immunity in the proatherogenic properties of the beige mutation in mice, we generated triple mutants in which beige, LDLr−/− mice were crossed with recombines activator gene 1 (Rag1)-deficient mice. Targeted disruption of Rag1 in mice results in a total inability to initiate V(DJ) rearrangement, resulting in a failure to generate mature antigen-specific T, NK/T, and B lymphocytes.

Likewis, compared with Rag1−/−, LDLr−/− control mice, triple-mutant beige, Rag1−/−, LDLr−/−; Pfp−/− mice had exacerbated atherosclerosis, suggesting that the proatherogenic properties of the beige mutation are independent of either NK cell cytolytic function or T and B lymphocyte function.

Methods

Animals

LDL receptor–deficient mice backcrossed onto a C57Bl/6 background (LDLr−/− mice) were purchased from Jackson Laboratories (Bar Harbor, Me) and bred in-house. Double-mutant mice were generated by crossing LDLr−/− mice with C57Bl/6-Lyst+/−, LDLr−/−, Lyst+/− mice, or C57Bl/6-Ldlr−/− mice, or C57Bl/6-Ldlr−/−, Rag1−/− mice. Rag1−/−, LDLr−/− mice were generated from mice that were C57Bl/6-Ldlr−/−; Rag1−/−, LDLr−/−, Pfp−/−, LDLr−/−, Pfp−/− mice. Reverse transcription (RT)–polymerase chain reaction (PCR) was used to genotype mice for the LDLr−/− and Pfp−/− mutations (see below), whereas the beige mutation was phenotyped by its dark gray coat color. Rag1−/−, LDLr−/− mice were phenotyped by fluorescence-activated cell sorting analysis (FACSscan, Becton-Dickinson) of CD3+ lymphocytes (described below). Mice were weaned at 4 weeks of age and fed ad libitum a standard mouse chow diet (Purina 7012, Harlan Teklad). Between 8 and 10 weeks of age, all mice were fed an atherogenic diet (hereafter referred to as high-fat diet [HFD]) for 16 weeks to induce atherosclerosis. All mice were housed under identical conditions in a sterile mouse facility, and all procedures were performed in accordance with institutional guidelines.

Twelve male beige, LDLr−/− mice, 12 Pfp−/−, LDLr−/− mice, and 16 LDLr−/− mice were used for the double-mutant study. Twelve beige, Rag1−/−, LDLr−/− mice and 16 Rag1−/−, LDLr−/− mice were used for the triple-mutant study. Because they were severely immunocompromised, animals in the triple-knockout study were treated prophylactically with an antibiotic, sulfamethoxazole–trimethoprim (Sulfadrin pediatric suspension, 65 mg/kg per day, Alpharma), for 1 week before and 16 weeks during HFD feeding to improve their health and survival. Sulfadrin was administered via the drinking water (5 mL per 500 mL water), protected from light, and replaced every 1 to 2 weeks.

Flow Cytometry

Cell pellets obtained from 0.1 mL peripheral blood drawn from the retro-orbital sinus were washed in PBS containing 2% FBS (2% PBS–BBS). After the cell suspension was incubated at 4°C with 1 μL anti-murine Fc receptor monoclonal antibody (Fc block, CD16/32, clone 2.4G2, Pharmingen), the cells were stained for 30 minutes at 4°C with fluorescein-5-isothiocyanate–labeled anti-mouse CD3 (clone 145-2C11, Pharmingen). A flow cytometer was used by using a FACScan flow cytometer (Becton-Dickinson) and analyzed by using CELLQuest software (Becton-Dickinson). Lymphocytes were gated by forward and side scatter, and fluorescent data were collected on 10,000 events within the lymphocyte gate.

Reverse Transcription–Polymerase Chain Reaction

Total RNA for RT-PCR was isolated from whole blood by using the RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. Random primers (0.05 μg/μL, Gibco-BRL) were annealed to 2 μg RNA in a thermocycler (MJ Research) set for 10 minutes at 95°C. Reverse transcriptase reaction buffer, dithiothreitol (0.01 mol/L final), dNTP (0.5 mmol/L final), RNasin (4 U, Promoega), and Superscript II (200 U, Gibco-BRL) were added to each sample and heated to 37°C for 50 minutes, 42°C for 10 minutes, and 95°C for 5 minutes. cDNAs were analyzed by PCR for LDL receptor and perforin expression and compared with G3PDH expression. Each reaction mixture contained 5 μL enzyme reaction buffer containing final concentrations as follows: Tris-HCl, pH 8.8 (67 mmol/L), (NH4)2SO4, (16.6 mmol/L), MgCl2, (7.6 mmol/L), BSA, (1.7 mg/mL), dNTP (1.0 mmol/L), dimethyl sulfoxide (0.704 mol/L), 0.5 μmol/L upstream primer (5′-GCTGCAAATCATCATATGC-3′ [mutLDLR1, Operon]) or 5′-GCTAAGTGACATGGATTTAC-3′ [mutPfp5, Operon]), 0.5 μmol/L downstream primer (5′-ACTGAAAATCATACGTC-3′ [mutLDLR2, Operon]) or 5′-GGGATTGTATTTGTCTCACACA-3′ [mutPfp6, Operon]), and 2.5 U Taq DNA polymerase. Three microliters of cDNA for LDLr−/− or 2 μL of cDNA for Pfp−/−, and 32 μL of 15% glycerol were added to 15 μL of the reaction mixture and placed in the thermocycler set as follows: After the initial denaturation step at 94°C for 5 minutes, samples were subjected to 35 cycles for 1 minute at 94°C, 1 minute at 58°C, and 1 minute at 72°C. After a final extension of 7 minutes at 72°C, samples were stored at 4°C. PCR products were resolved on a 3% NuSieve (3:1) agarose gel (FMC Bioproducts) containing 0.5 μg/mL ethidium bromide and were run at 100 V for 45 minutes in a tris-acetate EDTA running buffer and observed by UV light. The size of the RT-PCR product in the wild type was 275 bp, and there was an absence of product in the mutant.

Analysis of Atherosclerosis

Atherosclerosis in the aorta and the arterial root was assessed as described previously after mice had consumed the HFD for 16 weeks. Plasma total cholesterol was monitored throughout the 16-week protocol. Mice were fasted for 6 hours, and venous blood was drawn from the retro-orbital sinus into a heparinized capillary tube. Plasma was isolated by centrifugation at 5000 g for 10 minutes at 4°C and stored at −70°C. Total plasma cholesterol was measured at 0, 4, 8, and 16 weeks by a colorimetric method (Sigma Chemical Co.).

Histological Analysis

Frozen aortic root serial sections from 36 additional male LDLr−/− mice fed the HFD for 3, 6, or 16 weeks were stained for asialo-GM-1 antibody (Wako) on the spleen sections revealed that the antibody concentration of 1:12 000 dilution used in conjunction
with the tyramide signal amplification (TSA) system (NEN Research Products) identified only the NK cells and not the macrophages, which were identified with 1:10 000 dilution of MOMA-2 (Srotec) antibody used with TSA. Thus, spleen as well as aortic root sections were blocked for 30 minutes in 5% normal goat serum and then stained for 2 hours at room temperature with rat anti-mouse MOMA-2 (1:10 000) and rabbit anti-mouse asialo-GM-1 (1:12 000) antibodies to identify macrophages and NK cells, respectively. Endogenous peroxidase activity was quenched by incubation for 1 minute with Peroxox-Block (Zymed) and incubation with 1:1000 biotin-goat anti-rat IgG (MOMA-2 sections) or 1:1000 biotin-goat anti-rabbit IgG (asialo-GM-1 sections) for 90 minutes. The sections were incubated for 30 minutes with Vectastain ABC Elite solution (Vector Laboratories), 5 minutes with 1:100 solution of TSA system, and 30 minutes with Vectastain solution. The sections were then developed with AEC (Vector), counterstained for 1 minute in Gill’s hematoxylin No. 1, and mounted by use of aqueous mounting media.

**Cytolytic Activity**

After 16 weeks of consuming the HFD, the NK cell–specific cytolytic activity was assessed by a 4-hour $^{51}$Cr release assay. Mice were treated 24 hours before euthanasia with 100 µg IP Poly:IC (Sigma). Spleens were removed before perfusion-fixation, and splenocytes were enriched by Histopaque-1083 (Sigma). Spleens were removed before perfusion-fixation, and splenocytes were enriched by Histopaque-1083 (Sigma). Cells sedimenting at the interface between the Histopaque and plasma were enriched by Histopaque-1083 (Sigma). Cells sedimenting at the interface between the Histopaque and plasma were enriched by Histopaque-1083 (Sigma). Cells sedimenting at the interface between the Histopaque and plasma were enriched by Histopaque-1083 (Sigma). Spleens were removed before perfusion-fixation, and splenocytes were enriched by Histopaque-1083 (Sigma). Spleens were removed before perfusion-fixation, and splenocytes were enriched by Histopaque-1083 (Sigma). Cells sedimenting at the interface between the Histopaque and plasma were enriched by Histopaque-1083 (Sigma). Cells sedimenting at the interface between the Histopaque and plasma were enriched by Histopaque-1083 (Sigma). Cells sedimenting at the interface between the Histopaque and plasma were enriched by Histopaque-1083 (Sigma).

Isolated splenocytes for 4 hours in a humidified incubator (37°C and 5% CO$_2$). $^{51}$Cr release was determined in 25 µL culture supernatant by γ-sciillation (Packard Instruments Co). Results were expressed as the percentage of lysis according to the following equation: % lysis = (experimental release - spontaneous release)/(maximum release - spontaneous release) x 100. The data were normalized for total splenocyte number.

**Statistical Analysis**

All results were expressed as mean±SD, except where noted. Data were analyzed by the Mann-Whitney test with use of the Statview SE+ statistics package (SAS Institute Inc). A value of $P<0.05$ was considered significant.

**Results**

To verify that NK cells are present in lesions of LDLr$^{-/-}$ mice, immunohistochemistry with use of the asialo-GM-1 antibody was performed on aortic root serial sections. Asialo-GM-1 glycolipid is not selective for NK cells, in that it is present also in decreased amounts in thymic epithelial cells, Thy-1$^+$ dendritic epidermal cells, and macrophages. Therefore, preliminary studies to establish staining conditions and the specificity of controls were performed on spleen sections of C57Bl/6 mice infected with mouse cytomegalovirus (MCMV), which is known to recruit NK cells to the spleen.

With careful titration of the asialo-GM-1 antibody, NK cells were present in aortic root sections from LDLr$^{-/-}$ mice fed the HFD for 3 and 6 weeks. Panels A and B show distinct red asialo-GM-1–positive NK cells in spleens of MCMV-infected mice. Panels E and F show that infected spleens contained few NK cells. Panels C and D show that infected spleens had higher numbers of MOMA-2–positive macrophages than did uninfected spleens in panels G and H. NK cells were present in aortic root sections from LDLr$^{-/-}$ mice fed the HFD for 3 weeks (panels I through K) and for 6 weeks (panels L through Q). Compared with NK cells, MOMA-2–positive macrophages in aortic root sections from LDLr$^{-/-}$ mice fed the HFD for 3 weeks (panels L and M) and for 6 weeks (panels R through T) had a different staining pattern. Original magnifications were as follows: ×40 (panels A, C, E, and G), ×100 (panels B, D, F, and H), and ×250 (panels I through T).
cell–specific staining was obtained in spleens of infected and uninfected mice. Figure 1A and 1B shows red asialo-GM-1–positive NK cells from an MCMV-infected spleen, whereas an uninfected spleen had few NK cells (Figure 1E and IF). MOMA-2–positive macrophages were present in infected (Figure 1C and 1D) and uninfected (Figure 1G and 1H) spleens, although they were found in higher number in MCMV-infected spleens. Importantly, the asialo-GM-1–positive NK cells were distinct from MOMA-2–positive macrophages in the splenic serial sections, demonstrating that cross-reactivity between asialo-GM-1– and MOMA-2+ cells was minimal with this staining protocol.

Frozen aortic root serial sections from LDLr−/− mice that were fed the HFD for 3, 6, and 16 weeks were examined for MOMA-2–stained macrophages and asialo-GM-1–stained NK cells. NK cells were present in aortic root sections from LDLr−/− mice fed the HFD for 3 weeks (Figure 1I through 1K) and for 6 weeks (Figure 1N through 1Q). No NK cells were observed at 16 weeks (data not shown). As in the spleen serial sections, the staining for NK cells in the aortic root was distinct from the staining for MOMA-2–positive macrophages from LDLr−/− mice fed the HFD for 3 weeks (Figure 1L and 1M) and for 6 weeks (Figure 1R through 1T). These results illustrated the presence of NK cells in early, but not late, atherosclerotic lesions.

To understand the role of NK cell–mediated cytolysis in atherogenesis, we crossed NK cell–defective Lyst<sup>aggr</sup> mice and Pfp−/− mice with LDLr−/− mice (creating beige, LDLr−/− and Pfp,LDLr−/− mice, respectively). Because LDLr−/− mice have minimal lesions 3 and 6 weeks after consuming the HFD, atherosclerosis was quantified 16 weeks after the mice had consumed the atherogenic diet. Compared with LDLr−/− mice, aortic lesion area and aortic root lesion area were unchanged in Pfp−/−,LDLr−/− mice and significantly increased in beige,LDLr−/− mice (P<0.05, Figure 2A and 2B). It is interesting to note that Pfp−/−,LDLr−/− mice had significantly increased plasma total cholesterol at week 16 (please see online Figure I, available at http://atvb.ahajournals.org), yet the quantification of atherosclerosis was no different in these mice compared with control mice. Compared with control mice, beige,LDLr−/− mice had significantly decreased plasma total cholesterol at week 8, and the lesion area was significantly greater.

The cytolytic activity of isolated splenocytes was measured by <sup>51</sup>Cr release. The NK cell–susceptible Yac-1 mouse lymphoma cells were used as the <sup>51</sup>Cr-labeled target cells. As expected, compared with total splenocytes of control LDLr−/− mice, total splenocytes of beige,LDLr−/− and Pfp,LDLr−/− mice after consuming HFD for 16 weeks had severely impaired cytolytic activity (Figure 3). Although the beige,LDLr−/− mice and the Pfp−/−,LDLr−/− mice had defective NK cell cytolytic activity, only the beige,LDLr−/− mice had exacerbated atherosclerosis. These results indicate that the Lyst<sup>aggr</sup> mutation may affect atherosclerosis in a manner that is independent of impaired NK cell cytotoxicity.

To examine the role of acquired immunity on the effect of the beige mutation on atherosclerosis, we crossed beige mice with Rag1−/−,LDLr−/− mice to generate triple-mutant mice. Plasma total cholesterol values of beige,Rag1−/−,LDLr−/− mice were greater than those of Rag1−/−,LDLr−/− control mice (please see online Figure II, available...
at http://atvb.ahajournals.org), and lesion area in the aortic sinus was significantly increased (P<0.014) compared with lesion area in the Rag1−/−,LDLr−/− control mice (Figure 4A). Lesion area in the aorta was greater in beige,Rag1−/−,LDLr−/− mice, but this difference did not achieve significance (Figure 4B). These results support the conclusion that the beige mutation in LDLr−/− mice has proatherogenic properties. Moreover, this proatherogenic phenotype was observed in mice with severely defective acquired immunity (beige,Rag1−/−,LDLr−/− mice) and in mice with intact acquired immunity (beige,LDLr−/− mice).

Discussion

We identified NK cells in aortic root lesions of LDLr−/− mice. Compared with Rag1−/−,LDLr−/− mice, beige,Rag1−/−,LDLr−/− triple-mutant mice also had increased lesions. Therefore, the beige mutation has proatherogenic properties that are independent of NK cell cytotoxicity and acquired immunity.

The beige mutation in humans (CHS) is a rare autosomal recessive disorder in which patients present with ocucutaneous albinism, bleeding disorders, and recurrent infection (for review, see Introne et al15). The hallmark of CHS is the presence of large cytoplasmic granules in circulating granulocytes. Beige mice demonstrate characteristics similar to those of human CHS patients, including pale coat color, high rates of infection, and giant granules, and these mice are considered to be the animal homologue of CHS.13,14 The mouse gene is named lysosome trafficking regulator, or Lyst. Lyst is localized to the proximal end of mouse chromosome 13 and encodes a 429-kDa protein that is expressed in most tissues, although the function remains elusive.20,21 The beige/CHS mutation affects cellular melanosomes, lysosomes, and platelet dense bodies, all of which arise, in part, from the trans-Golgi network. Deficiency in the beige/CHS protein results in perinuclear clustering of excessively large granules, whereas overexpression results in abnormally small lysosomes that localize to the cell periphery.22 Increasing evidence suggests that the clinical symptoms of CHS patients can be explained by vesicle secretion malfunction; however, there is disagreement over the origin of the giant lysosomes. Several models have been proposed to explain the relationship between Lyst and enlarged lysosomes: (1) Lyst acts as a negative regulator of lysosomal fusion;14 (2) Lyst is a positive regulator of vesicle fission22,23; and (3) Lyst regulates protein transport to late endosomes.24 In the review by Introne et al, it was suggested that the gene defect involves impaired exchange of membrane material between the trans-Golgi network and late endosomes and that this process is mediated by the beige/CHS protein, presumably because of its association with microtubules.15

The NK cell abnormality has been suggested to be primarily responsible for the defective cellular immunity and the accelerated phase, which ultimately leads to the demise of CHS patients. However, the beige mutation is not limited to NK cells and affects other cell types, such as monocytes/macrophages, which are important in atherosclerosis. Giant perinuclear granules in the cytoplasm of monocytes as well as neutrophils have been suggested to impose a mechanical impediment to these cells, preventing their infiltration of the endothelium and emigration into tissues.25 Furthermore, beige mice have an increased susceptibility to Mycobacterium avium infection that is, in part, due to the impaired chemotaxis in response to decreased macrophage inflammatory protein-2 and macrophage chemotactic protein-1 expression in the early stages of infection.26,27

In conclusion, we have provided evidence that although NK cells are present in mouse lesions, NK cell–mediated cytolytic activity does not play a significant role in atherosclerosis. Because NK cells also produce cytokines, these studies do not rule out a role of NK cell cytokine production in atherosclerosis. Moreover, we documented that LDLr−/− mice with the beige mutation had exacerbated atherosclerosis,
which occurred independently of NK cell cytolytic activity and/or T and B lymphocyte function. Finally, the present study suggests that lysosomal trafficking and vesicle maturation may play a key role in atherosclerosis.

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