Effect of \(\gamma\)-Irradiation and Bone Marrow Transplantation on Atherosclerosis in LDL Receptor–Deficient Mice

Natalie K. Schiller,* Nobuhiko Kubo,* William A. Boisvert, Linda K. Curtiss

Abstract—Bone marrow transplantation (BMT) is commonly used to study the participation of bone marrow–derived cells in atherosclerosis. To determine the effect of this methodology on lesions, 16 male low density lipoprotein (LDL) receptor knockout (LDLr−/−) mice were reconstituted with bone marrow from syngeneic LDLr−/− mice after 10 Gy \(\gamma\)-irradiation and compared with 12 male LDLr−/− littermates that did not undergo BMT (no-BMT group). Mice were fed a high fat diet (HFD) for 16 weeks to induce atherosclerosis. Sixteen additional LDLr−/− mice underwent BMT, and 12 male LDLr−/− mice that did not undergo BMT were fed a chow diet for 56 weeks. Thoracic aorta lesion areas were smaller in BMT mice than in no-BMT mice fed the HFD (\(P<0.0001\)). In contrast, aortic root lesion areas were greater in the BMT mice fed the HFD (\(P<0.0001\)) as well as in those fed the chow diet (\(P=0.0001\)). Abdominal aorta free cholesterol and cholesteryl ester mass were minimal in all groups studied. Aortic root lesions from all no-BMT mice were densely collagenous and encapsulated by a cellular cap, whereas lesions in the BMT mice contained lipid cores and minimal collagen staining. Although the reason for these differences in lesion size and composition remains unresolved, this study suggests that multiple parameters of lesion formation should be examined to assess atherosclerosis. (Arterioscler Thromb Vasc Biol. 2001;21:1674-1680.)

Key Words: bone marrow transplantation \(\gamma\)-radiation macrophages collagen hyperlipidemia

The LDL receptor knockout (LDLr−/−) mouse is a particularly suitable model for studying atherosclerosis. These mice develop atherosclerotic lesions predominantly in the aortic sinus when they are fed a chow diet, whereas they develop extensive lesions throughout the aortic root and the length of the entire aorta when they are fed a high fat diet (HFD).1 Atherosclerosis in mice has been assessed by cross-sectional analysis of the aortic sinus,2 and morphometric methods are also available to quantify the extent of lesion involvement in the entire aortic tree by measuring stained en face dissected aortas.3 More recently, free cholesterol and cholesteryl ester mass measurements of dissected, homogenized, and lipid-extracted aortas have been used to assess atherosclerosis.4

Lethal total body \(\gamma\)-irradiation of atherosclerosis-prone mice followed by bone marrow reconstitution from donors with transgenic alterations in the innate and acquired immune systems has been used experimentally to identify the role of bone marrow–derived cells in atherosclerosis.5 Although in all cases the experimental mice are compared with control mice that have undergone comparable irradiation and reconstitution with syngeneic wild-type bone marrow, the direct effects of lethal total body irradiation and syngeneic bone marrow transplantation (BMT) on atherosclerosis have not been examined. In this methodological study, we examined the effects of this experimental model on atherosclerosis in male LDLr−/− mice fed either an HFD for 16 weeks to induce rapid lesion formation or a chow diet for 56 weeks to examine more moderate lesion formation. We found that atherosclerosis in BMT LDLr−/− mice had a distinct collagen content and macrophage distribution that was not observed in the mice that did not undergo BMT (no-BMT group). Furthermore, we found that multiple measurements commonly used to assess atherosclerosis in mice did not give analogous results, suggesting that (when possible) multiple parameters should be used to quantify atherosclerosis in LDLr−/− mice.

Methods

Animals

LDL receptor–deficient mice backcrossed onto a C57Bl/6 background (LDLr−/− group) were purchased from Jackson Laboratories (Bar Harbor, Me) and bred in-house. The mice were weaned at 4 weeks and fed ad libitum a standard mouse chow diet (Purina 7012, Harlan Teklad) or an atherogenic diet (HFD) containing the following: 15.8% fat, 1.25% cholesterol, and no cholic acid (No. 94059, Harlan Teklad).4 The mice were housed 4 per cage in autoclaved filter-topped cages with autoclaved water and kept on a 12-hour light-dark cycle. All procedures were performed in accordance with institutional guidelines.

Irradiation and BMT

Thirty-two male LDLr−/− mice (8 weeks old) were subjected to 10-Gy lethal total body \(\gamma\)-irradiation to eliminate endogenous bone
marrow stem cells and bone marrow–derived cells. This dose was chosen because it completely ablates the rapidly dividing bone marrow–derived cells. Bone marrow cells used for repopulation were extracted from the femur and tibia of 4 male LDLr−/− and 4 female LDLr−/− mice, as previously described. All irradiated mice were injected intravenously with 3 × 10^6 bone marrow cells from either male or female LDLr−/− mice. Hereafter, BMT will indicate bone marrow transplantation, which is an all-inclusive term for the accompanying γ-irradiation as well as the bone marrow reconstitution. In the short-term study, 16 mice were fed chow for 4 weeks after BMT to allow for bone marrow reconstitution and then switched to the HFD for an additional 16 weeks to induce rapid lesion formation. In the long-term study, 16 mice were maintained after BMT on the chow diet for 56 weeks before euthanasia. Two groups of 12 age-matched male LDLr−/− littermates that did not undergo BMT served as the no-BMT control group and were fed either the HFD or chow diet. These mice were handled and housed under conditions identical to those for the BMT mice. Figure 1 illustrates the experimental design.

Periodically, mice were fasted for 6 hours, anesthetized with methoxyflurane, and weighed, and venous blood was drawn from the retro-orbital sinus into a heparinized capillary tube. Twenty microliters of peripheral whole blood was hemolyzed in 180 μL of 1% acetic acid, and total white blood cells were counted by use of a hemocytometer. Plasma was isolated from the remaining venous blood by centrifugation at 5000g for 10 minutes at 4°C and stored at −70°C. Plasma total cholesterol and triglyceride levels were measured by a colorimetric method with use of a kit from Sigma Chemical Co.

**Analysis of Atherosclerosis**

At euthanasia, animals were perfused with PBS, followed by formal-sucrose (4% paraformaldehyde and 5% sucrose in PBS, pH 7.4). The entire mouse aorta was dissected from the proximal ascending aorta to the bifurcation of the iliac artery by using a dissecting microscope. Adventitial fat was removed, and the thoracic aorta was dissected from the right common carotid artery to the superior mesenteric artery. The thoracic artery was opened longitudinally, pinned flat onto black dissecting wax, stained with Sudan IV, and photographed at a fixed magnification. The photographs were digitized, and total aortic area and aortic lesion area were calculated by using Adobe Photoshop 5.0.2 and NIH Scion Image software. The results were reported as percentage of the total thoracic aorta area that contained lesions.

As a second assessment of atherosclerosis, lesions of the aortic root were analyzed as previously described. The top half of the heart was removed and immersed in formal-sucrose for 6 hours. The hearts were embedded in OCT (Sakura Finetek USA) and stored at −70°C until sectioning. Serial sections (10 μm in thickness) were cut through a 250-μm segment of the aortic root, where all 3 valve leaflets were present. For each mouse, 4 sections separated by 40 μm were examined. Each section was stained with oil red O, counterstained with Gill’s hematoxylin #1 (Fisher Scientific), and digitzed, and the total lesion area was quantified. Lesion area included the entire intima, including lipid cores and fibrotic components.

The dissected aortas from the superior mesenteric artery to the iliac bifurcation were homogenized and subjected to lipid extraction as described previously. Aortic choleseryl esters and free cholesterol mass were separated by thin-layer chromatography and quantified.

**Histological Analysis**

To further characterize the morphological and cellular composition of aortic root sections, immunohistochemical analysis was performed. Aortic root lesion sections were stained immunohistochemically for MOMA-2 to identify macrophages. For mouse macrophage antigen (MOMA)-2 staining, cryosections were fixed to the glass microscope slide by incubation in −20°C acetone for 2 minutes and rehydration in PBS for 5 minutes. Nonspecific staining was blocked by using 5% normal rabbit serum. Sections were incubated 1 to 2 hours at room temperature with polyclonal rat anti-mouse MOMA-2 antibody (1:1000, Serotec Ltd). Endogenous peroxidase was quenched with a blocking agent (Zymed). The biotinylated rabbit antibody (1:1000, Chemicon International, Temecula, CA) was applied, followed by streptavidin–horseradish peroxidase (1 μg/mL) and 30-minute exposure to Vectorstain ABC Elite solution (Vector Laboratories), followed by 3,3′-diaminobenzidine tetrahydrochloride (DAB, Vector Laboratories). Masson’s trichrome was used to identify collagen within the aortic root sections. Cryosections were placed in Bouin’s fixative for 1 hour at 56°C and cooled for 10 minutes. Sections were placed in Weigert’s iron hematoxylin solution for 10 minutes and washed for another 10 minutes. Sections were then placed in filtered Biebrich scarlet-acid fuchsin solution for 15 minutes and rinsed in 1% acetic acid. Slides were dehydrated and mounted. Nuclei stained black; cytoplasm, keratin, and muscle fibers stained red; and collagen and mucin stained blue.
Statistical Analysis
All results were expressed as the mean±SD, except where noted. Data were analyzed by Mann-Whitney test with the use of the Statview SE+ statistics package (SAS Institute Inc). A value of P<0.05 was considered significant.

Results
Our experimental design involved the comparison of male nonirradiated LDLr−/− (no-BMT) mice with male irradiated and bone marrow reconstituted (BMT) mice fed either chow or HFD. Half the male BMT recipients received bone marrow cells from male mice, and the other half received bone marrow cells from female mice. The female bone marrow was included so that, if necessary, BMT mice could be distinguished from no-BMT mice by real-time reverse transcription–polymerase chain reaction amplification of the Y chromosome marker, Sry (a testis-determining gene) from the harvested tissues. However, identification of female-derived cells to distinguish BMT mice was not necessary and therefore not performed. Statistics were performed to determine whether significant differences existed between the BMT mice that received female marrow and BMT mice that received male marrow. In the analyses performed, only plasma total cholesterol at the time of euthanasia in the HFD study significantly differed between BMT mice that received male bone marrow and BMT mice that received female bone marrow (P=0.009 for male bone marrow; P=0.04 for BMT vs no-BMT group). Bottom, In the long-term study, the animals received only chow and were euthanized at 56 weeks. Data are mean±SD. Solid square indicates BMT group (n=13); solid circle, no-BMT group (n=12). +P=0.0003 for BMT vs no-BMT group.

A comparison of BMT versus no-BMT mice revealed no differences in total plasma cholesterol levels with either diet (Figure 2). Moreover, total cholesterol levels (averaging 270 mg/dL) were not significantly different before or after BMT (week 0 versus week 4), suggesting that BMT had no effect on plasma total cholesterol. In the short-term study, BMT mice fed the HFD for 4 weeks had cholesterol levels of 1050±260 mg/dL compared with levels of 1030±190 mg/dL in the no-BMT mice (Figure 2, top). After 16 weeks on the HFD, BMT mice had cholesterol levels of 1117±260 mg/dL compared with levels of 1133±220 mg/dL in no-BMT mice. Thus, there were no significant differences for the mean time-matched cholesterol levels between BMT and no-BMT mice. In the long-term study, mice fed the chow diet maintained average plasma cholesterol levels of <400 mg/dL for 56 weeks (Figure 2, bottom). Plasma triglyceride levels of all the mice remained <140 mg/dL throughout the study (data not shown).

To determine the effect of BMT on atherosclerosis, we first used a commonly reported measurement of atherosclerosis in LDLr−/− mice, the surface area of lesions in the thoracic aorta. After dissection, no macroscopic changes or malformations were observed in the entire aortas of either BMT or no-BMT mice. There were visible Sudan IV–stained lesions on the luminal surface of the thoracic aortas of all mice. BMT mice had fewer lesions in the arch and more along the intercostal arteries, whereas the no-BMT mice had large lesion areas in the arch and fewer in the intercostal arteries. Cross sections of the aorta at the arch revealed that lesions in no-BMT mice fed HFD were larger and more complex than were the lesions of BMT mice (Figure 3). On quantification,
lesion areas of BMT mice fed the HFD, reported as the percentage of the total area of the thoracic aorta, were significantly less \( (P<0.0001, \text{Figure 4A}) \), whereas the lesion areas of the BMT mice fed chow were not significantly less \( (\text{Figure 5A}) \).

We also studied the effect of BMT on atherosclerosis by examining another commonly assessed anatomic site, the aortic root lesions.\(^9\) In contrast to our measurements of the thoracic aorta lesions, the aortic root lesion areas gave very different results. Aortic root lesion areas of mice fed the HFD were 2-fold greater in BMT mice than in no-BMT mice \( (P<0.0001, \text{Figure 4B}) \). Furthermore, the aortic root lesion areas were also greater in BMT chow-fed mice than in the no-BMT chow-fed mice \( (\text{Figure 5B}) \). Interestingly, the aortic root lesion areas of the no-BMT LDLr\(^{-/-}\) mice fed chow for 56 weeks were similar to the aortic root lesion areas of mice fed the HFD for 16 weeks. However, the BMT LDLr\(^{-/-}\) mice fed the HFD had almost twice as much lesion area as the BMT chow-fed mice.

The final assessment of atherosclerosis used measurements of cholesterol accumulation in the dissected aorta (Figure 6). Cholesteryl ester mass and free cholesterol mass in the abdominal aorta were measured by thin-layer chromatography after lipid extraction.\(^4\) There were no significant differences between either cholesterol ester or free cholesterol in the vessels from BMT and no-BMT mice fed the HFD or chow diet. However, it should be noted that compared with aortas from mice on the chow diet, aortas from all mice on the HFD had greater cholesteryl ester and free cholesterol levels.
To further characterize lesion differences in mice, cellular and extracellular composition of aortic root lesions were examined. Figure 7 illustrates the distribution of macrophages and collagen within the aortic root lesions of mice fed the HFD. BMT and no-BMT mice had aortic root lesions with distinct staining distributions of macrophage-specific MOMA-2 antibody and collagen. The aortic root lesions of no-BMT mice were densely collagenous, with abundant collagen encapsulated by a thick cellular layer colocalized with limited MOMA-2–positive macrophages. This minimal infiltration of macrophages probably accounted for the less foamy appearance of the aortic root lesions in the no-BMT mice. In contrast, the aortic root lesions of the BMT mice appeared more foamy and lipid-filled and had a greater degree of macrophage infiltration. MOMA-2–positive macrophages were found at the perimeter and were also diffusely distributed throughout the lesion. In place of collagen, there were large lipid cores in the aortic root lesions and a disorganized cellular cap.

This distinction between the lesions of BMT and no-BMT mice was evident in the chow-fed mice as well (Figure 7). No-BMT mice fed a chow diet for 56 weeks had aortic root lesions that stained lightly for collagen and were encapsulated by a thin cellular layer at the lumenal surface. This layer of cells at the lumenal surface stained for MOMA-2. The aortic root lesions were thicker and more extensive in BMT mice than in no-BMT mice, and they also contained some collagen. However, the collagen was more dense at the periphery rather than throughout the interior of the lesion, as observed in the aortic root lesions of the no-BMT mice. BMT mice also had a thin, loosely organized cellular cap near the lumenal surface.

**Discussion**

Studies of diet-induced hyperlipidemia in LDLr−/− mice have reported that morphometric analysis of lesions in the aortic root and the aorta can be examined to quantify disease severity. These methods have been reported to provide complementary information on the degree and distribution of atherosclerosis. A correlation between multiple measurements has also been demonstrated in studies using irradiation and BMT. However, the present methodological study showed that under some conditions, such as a comparison of
BMT and no BMT, these measurements can give contradictory results. Compared with no BMT in the littermate control group, BMT exacerbated atherosclerosis within the aortic root yet appeared to inhibit lesion progression across the surface of the thoracic aorta. In the present study, we have included a representative cross section of the aortic arch from BMT and no-BMT mice to illustrate the point that BMT mice had a greater lesion area in the aortic root but a smaller lesion area in the aorta, whereas no-BMT mice had a greater lesion area in the aortic root but a smaller lesion area in the aorta (Figure 4). Direct comparisons with the same method of measurement (ie, cross-sectional area) within the aortic root and the aorta are not possible because of the length of the vessel and the disparate distribution of lesions throughout the entire aorta. Cross-sectional analysis of the aorta would result in bias because of the part of the aorta used in the analysis. The length of the aorta limits its use in this manner because of the number of sections that would be required to achieve an appropriate sampling of the lesion. We have performed regression analysis for the lesion areas in the thoracic aorta and aortic root as well as aortic cholesterol and cholesteryl ester mass and found that the $R^2$ values for every comparison, regardless of diet, were <0.37 (data not shown). This suggests that no correlations existed between the different anatomic sites and the methodologies that we used in assessing atherosclerosis. The reasons for the discrepancies in lesion area remain unresolved. However, the results illustrate the importance of measuring multiple parameters of lesion formation to determine the effects of various treatments on the progression of diet-induced atherosclerosis, unless it has been previously demonstrated that the parameters are correlated.

The LDLr$^{-/-}$ mouse, a common model suitable for studying atherosclerosis, develops small atherosclerotic lesions predominantly in the aortic sinus when fed a chow diet. These mice develop extensive lesions throughout the aortic root area characterized by disturbed flow, whereas the aorta was characterized by laminar flow. The vascular endothelium can respond to fluid mechanical forces generated by pulsatile blood flow and is able to distinguish between disturbed and laminar flow patterns. Hemodynamic flow influences endothelial cell proliferation and gene expression, which in turn can have an impact on atherosclerosis. In addition, radiation has a known detrimental effect on the endothelium. Therefore, the irradiated BMT mice may exhibit impaired endothelial responses in regions (such as the aortic sinus) that are more prone to the detrimental effects of disturbed flow and thus have increased atherosclerosis. Endothelial damage at a site predisposed to atherosclerosis in association with the disturbed flow characteristic of that site could account for the exacerbated atherosclerosis in the aortic root of BMT mice.

The lack of differences in aortic cholesteryl ester and free cholesterol between BMT and no-BMT mice despite significant differences in thoracic aorta lesion area may relate to the fact that lesion area was much reduced in the abdominal aorta compared with the thoracic aorta. This suggests that the lesion area in one portion of the vessel does not reflect the lesion area of another. Again, this could relate to differences in hemodynamic forces. However, it should be noted that all animals on the HFD had greater cholesteryl ester and free cholesterol than did the animals on the chow diet. This suggests that thin-layer chromatography can distinguish between larger differences in lesion size but is limited in its use in determining more subtle differences in lipid content.

The most provocative aspect of the present study was the distinct pathology of atherosclerosis in LDLr$^{-/-}$ mice receiving total body lethal irradiation and bone marrow reconstitution compared with the disease pathology of their no-BMT littermates. The aortic root lesions in BMT mice fed the HFD had large lipid cores that contained relatively little collagen. In contrast, the aortic root lesions of no-BMT mice had a dense collagen matrix that was encapsulated by a well-organized cellular layer. Smooth muscle cells are the primary producers of collagen within atherosclerotic lesions. Thus, irradiation and BMT may have influenced smooth muscle cell involvement, because the dense, organized cellular cap was noticeably absent in certain areas of the lesions of BMT mice.

Another striking difference between the morphology of the aortic root lesions in BMT and no-BMT mice was the macrophage distribution and degree of infiltration. The BMT mice had greater macrophage content throughout their lesions than did the no-BMT mice. $\gamma$-Irradiation affects endothelial cell integrity, which can lead to increased adhesion molecule expression and leukocyte adhesion. Thus, the difference in macrophage distribution and the degree of infiltration in the BMT mice may have been due to irradiation-induced effects.
endothelial cell injury or irradiation-induced macrophage proliferation and/or differentiation.

The present study shows that lethal total body irradiation and syngeneic bone marrow reconstitution, commonly used to study the role of bone marrow–derived cells in atherosclerosis, has a unique influence on atherosclerosis caused by the hyperlipidemia of mice fed an HFD as well as a chow diet. The present study also shows that BMT LDLr−/− mice have a disease that can be distinguished from the pathology of the no-BMT mice on the basis of collagen content and macrophage distribution within the aortic root. Furthermore, the present study demonstrates that techniques commonly used to assess murine atherosclerosis do not give the same results, and the study also reveals the importance of examining multiple parameters of lesion formation, especially when new causes or etiologies are examined.

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