Genes within the MHC Region have a Dramatic Influence on Radiation-Enhanced Atherosclerosis in Mice

Running title: Shi et al.; MHC and atherosclerosis

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Abstract:

Background - C3H/HeJ (C3H) mice develop much smaller atherosclerotic lesions than C57BL/6 (B6) mice when deficient in apolipoprotein E (apoE<sup>−/−</sup>) or fed an atherogenic diet. The two strains differ in H2 haplotypes with B6 having H2<sup>b</sup> and C3H having H2<sup>k</sup>. C3.SW-H2<sup>b</sup>/SnJ (C3.SW) is a congenic strain of C3H/HeJ in which H2<sup>k</sup> is replaced with H2<sup>b</sup>.

Methods and Results - We performed bone marrow transplantation and found that atherosclerosis-resistant C3.SW.apoE<sup>−/−</sup> mice reconstituted with bone marrow from either C3.SW.apoE<sup>−/−</sup> or B6.apoE<sup>−/−</sup> mice after lethal irradiation developed significantly larger atherosclerotic lesions than B6.apoE<sup>−/−</sup> mice receiving identical treatments and much larger lesions than C3H.apoE<sup>−/−</sup> mice reconstituted with syngeneic bone marrow. For syngeneic transplantation, C3.SW.apoE<sup>−/−</sup> mice exhibited a 21-fold increase in lesion size over C3H.apoE<sup>−/−</sup> mice (152,800±21,937 vs. 7,060±2,290 μm<sup>2</sup>/section) and a near 4-fold increase over B6.apoE<sup>−/−</sup> mice (40,529±4,675 μm<sup>2</sup>/section). C3.SW.apoE<sup>−/−</sup> mice reconstituted with syngeneic marrow exhibited enhanced lesion formation relative to those reconstituted with B6 marrow (152,800±21,937 vs. 107,000±9,374 μm<sup>2</sup>/section; P=0.067). Sublethal irradiation led to a 6-fold increase of lesion size in C3.SW.apoE<sup>−/−</sup> mice (9,795±2,804 vs. 1,550±607 μm<sup>2</sup>/section; P=0.008). Wild-type C3.SW mice reconstituted with apoE<sup>+/+</sup> or apoE<sup>−/−</sup> bone marrow developed significantly larger atherosclerotic lesions than C3H mice receiving identical treatments on an atherogenic diet.

Conclusions - These results indicate that gene(s) within the H2 region have a dramatic impact on radiation-enhanced atherosclerosis and their effect is conveyed partially through bone marrow-derived cells.

Key words: Atherosclerosis; radiation; major histocompatibility complex; H2 haplotype; mice
Introduction:

Radiotherapy is the most commonly used non-surgical modality for the treatment of malignant tumors and more than half of the long-term cancer survivors have experienced such treatment. Radiotherapy has also been used to treat proliferating, non-malignant diseases, such as severe hyperthyroidism, skeletal degenerative diseases, pigmented villonodular synovitis, keloid scar growth, and heterotopic ossification. However, patients receiving radiotherapy have an increased risk of developing atherosclerotic cardiovascular disease. There is accumulating evidence supporting radiation as a risk factor for atherosclerosis independent of such risk factors as hyperlipidemia, age, diabetes, hypertension, smoking, and lack of exercise. In humans, irradiation of the carotid arteries leads to atherosclerosis or accelerates progression of atherosclerosis. Experimental animal studies also show that irradiation increase atherosclerotic lesion size of high-fat fed C57BL/6 (B6) mice and apolipoprotein E-deficient (apoE<sup>-/-</sup>) mice and promotes monocyte transmigration into the subendothelial space and transformation into foam cells in hypercholesterolemic rabbits. However, it is not yet understood how radiation exacerbates the atherosclerotic condition.

Mouse strains B6 and C3H/HeJ (C3H) exhibit marked differences in their susceptibility to atherosclerosis. C3H mice develop much smaller atherosclerotic lesions than B6 mice when fed an atherogenic diet or deficient in apolipoprotein E (apoE<sup>-/-</sup>). Atherogenesis is a complicated process involving interactions among arterial wall cells, bone marrow-derived blood cells and lipids. A recent study by our group defined the arterial wall as a major source of the difference in atherosclerosis susceptibility as B6 aortic grafts developed significantly larger atherosclerotic lesions than C3H grafts when anastomosed between divided infrarenal aorta of identical F1 mice derived from the two strains. However, because the two parental strains exhibited much larger differences in atherosclerotic lesion sizes in non-transplanted aortas than...
in transplanted aortas, other components may also contribute. In the present study, bone marrow transplantation was undertaken to define the contribution of bone marrow-derived cells to atherosclerosis susceptibility. The findings excluded a significant role in this regard, but revealed a huge impact of genes within the H2 region on the progression of radiation-enhanced atherosclerosis.

Materials and methods:

Mice: C3H/HeJ (C3H), C3.SW-H2\(^b\)/SnJ (C3.SW), and B6.apoE\(^{-/-}\) mice were purchased from the Jackson Laboratory, Bar Harbor, ME. C3H.apoE\(^{-/-}\) mice and C3.SW.apoE\(^{-/-}\) mice were generated in our laboratory. The creation of C3H.apoE\(^{-/-}\) mice was previously reported.\(^{15}\) C3.SW.apoE\(^{-/-}\) mice were generated by initially crossing female C3H.apoE\(^{-/-}\) mice with male C3.SW mice, followed by three generations of sequential backcross mating to C3.SW mice and one generation of intercross mating with selection for the H2\(^b\) and apoE null alleles. The presence of the H2\(^b\) allele was determined by PCR using microsatellite markers D17Mit47 (36.35 Mb), D17Mit125 (36.63 Mb) and D17Mit148 (37.47 Mb), as reported by Xiao et al.\(^{16}\) All procedures were carried out in accordance with current National Institutes of Health guidelines and approved by our Institutional Animal Care and Use Committee.

Irradiation and bone marrow transplantation: Mice were irradiated from a \(^{137}\)Cs source (JL Shepherd Mark 1 \(^{137}\)Cs Irradiator) with a single dose of 0, 600 or 1,100 rads. The bone marrow transplantation procedure was performed as we previously described.\(^{17}\) Briefly, 5-week-old female recipient mice were lethally irradiated with a single dose of 1,100 rads and then reconstituted with \(10^7\) bone marrow cells harvested from the femurs and tibias of donor male mice. To evaluate the effect of radiation alone on atherosclerosis, 5-week-old female C3.SW.apoE\(^{-/-}\) mice were irradiated with a sublethal dose of 600 rads. ApoE\(^{-/-}\) mice were fed a
rodent chow diet throughout the entire experiment period. Wild-type mice were switched from a chow to an atherogenic diet containing 15% fat, 1.25% cholesterol, and 0.5% cholic acid (TD 90221, Harlan Teklad, Inc) 4 weeks after transplantation and fed the diet for 12 weeks, as previously reported.  

**Plasma lipid measurements.** Mice were fasted overnight before blood was collected through retro-orbital puncture under isoflurane inhalation. Plasma total cholesterol, HDL cholesterol, and triglyceride levels were measured with enzymatic assays as we previously described.  

**Aortic lesion analysis:** The method for quantification of atherosclerotic lesions in the aorta was as we previously reported. The lesion areas of five sections with the largest readings were averaged for each mouse and this average was used for statistical analysis.  

**Statistical Analysis.** Data were expressed as means ± SE, with “n” indicating the number of mice. Student’s T-test was used to test for differences between two groups in lesion size or plasma lipid levels. All T-tests were performed under the assumption of unequal variances. Differences were considered statistically significant at $P < 0.05$.  

**Results**  
We previously reported marked differences between B6.apoE$^{-/-}$ and C3H.apoE$^{-/-}$ mice in atherosclerotic lesion formation when fed a chow and Western diet.  

To determine whether cells of hematopoietic origin were a source of the difference in atherosclerosis susceptibility, we constructed C3.SW.apoE$^{-/-}$ mice, which carry the same H2$^b$ haplotype as B6.apoE$^{-/-}$ mice, and performed reciprocal bone marrow transplantation between the two strains. After transplantation, mice continued on the chow diet. Atherosclerotic lesions in the aortic root were measured 16 weeks after transplantation. Unexpectedly, lethally irradiated C3.SW.apoE$^{-/-}$ mice reconstituted with bone marrow from either B6.apoE$^{-/-}$ or C3.SW.apoE$^{-/-}$ mice developed
significantly larger atherosclerotic lesions than recipients reconstituted with bone marrow from the same donors ($P<0.0001$; Figure 1). For syngeneic marrow transplant recipients, C3.SW.apoE$^{-/-}$ mice had a lesion size of $152,800\pm21,937$ $\mu m^2/section$ (n=20), which was nearly 4-fold as larger as the lesion size of $40,529\pm4,675$ $\mu m^2/section$ in B6.apoE$^{-/-}$ mice (n=14; $P=0.000059$). C3.SW.apoE$^{-/-}$ mice reconstituted with syngeneic bone marrow exhibited a 50% increase in lesion size over those reconstituted with bone marrow from B6.apoE$^{-/-}$ mice (152,800$\pm$21,937 vs. 107,000$\pm$9,374 $\mu m^2/section$; n=20 and 23, respectively), although the difference did not reach statistical significance ($P=0.067$). In contrast, B6.apoE$^{-/-}$ mice reconstituted with bone marrow from C3.SW.apoE$^{-/-}$ mice developed a lesion size comparable to those reconstituted with syngeneic bone marrow (42,188$\pm$5,378 vs. 40,529$\pm$4,675 $\mu m^2/section$; n=16 and 14, respectively; $P=0.82$).

C3.SW.apoE$^{-/-}$ mice had a significantly higher total cholesterol level than B6.apoE$^{-/-}$ mice (404.2$\pm$9.0 vs. 352.0$\pm$23.2 mg/dl; $P=0.009$), although their HDL cholesterol (28.7$\pm$2.2 vs. 24.5$\pm$2.5 mg/dl; $P=0.09$) and triglyceride levels (110.1$\pm$8.1 vs. 101.8$\pm$8.8 mg/dl; $P=0.38$) were comparable (Figure 2). The higher non-HDL cholesterol level could be partially responsible for the increased lesion formation in C3.SW.apoE$^{-/-}$ mice.

When compared with C3H.apoE$^{-/-}$ recipients (n=12) reconstituted with syngeneic bone marrow, C3.SW.apoE$^{-/-}$$\rightarrow$C3.SW.apoE$^{-/-}$ mice exhibited a 21-fold increase in lesion size (152,800$\pm$21,937 vs. 7,060$\pm$2,290 $\mu m^2/section$; $P=0.0000025$) (Figure 3).

To determine whether radiation alone would increase lesion formation, female C3.SW.apoE$^{-/-}$ mice were irradiated with a sublethal dose of 600 rads. After irradiation, mice were maintained on the chow diet for 16 weeks. Compared to non-irradiated mice, irradiated mice exhibited a 6-fold increase in aortic lesion size (9,795$\pm$2,804 vs. 1,558$\pm$607 $\mu m^2/section$; n=5 and 9, respectively; $P=0.017$) (Figure 4). However, when compared to those C3.SW.apoE$^{-/-}$
mice lethally irradiated and reconstituted with syngeneic bone marrow, these mice developed much smaller aortic lesions (9,795±2,804 vs. 152,800±21,937 μm²/section). Sublethally irradiated C3.SW.apoE⁻/⁻ mice did not differ significantly from non-irradiated mice in total cholesterol (328.0±18.3 vs. 336.4±11.2 mg/dl), HDL cholesterol (13.6±1.1 vs. 19.2±1.2 mg/dl), and triglyceride levels (114.0±13.3 vs. 93.2 mg/dl) (Figure 5).

Wild-type C3.SW mice were totally resistant to atherosclerosis, developing no atherosclerotic lesions in the aortic root after 12 weeks on the atherogenic diet containing 15% fat, 1.25% cholesterol and 0.5% sodium cholate (Figure 6). In contrast, lethally irradiated C3.SW mice reconstituted with bone marrow from either C3.SW or C3.SW.apoE⁻/⁻ mice developed atherosclerotic lesions on the atherogenic diet. The difference in lesion size between mice reconstituted with apoE⁻/⁻ bone marrow and those reconstituted with apoE⁺/+ bone marrow was not statistically significant (9,183±2,605 vs. 9,033±1,717 μm²/section; n=9 and 15, respectively; P=0.50). When compared to C3H.apoE⁻/⁻→C3H.apoE⁺/+ mice (1,422±242 μm²/section, n=15), C3.SW.apoE⁺/+→C3.SW.apoE⁺/+ mice exhibited a 5-fold increase in aortic lesion size. C3H.SW.apoE⁻/⁻→C3H.SW.apoE⁺/+ mice exhibited a 6-fold increase in lesion size compared to C3H.apoE⁻/⁻→C3H.apoE⁺/+ mice (1,537±383 μm²/section, n=14).

Discussion

Although radiation is known to accelerate the development of atherosclerosis, the influence of genetic factors on this effect has not been reported. This study has clearly shown that the H2b haplotype is a major genetic determinant of radiation-enhanced atherosclerosis in C3.SW mice.

We previously demonstrated that the arterial wall was a major source of the marked difference between B6.apoE⁻/⁻ and C3H.apoE⁻/⁻ mice in atherosclerosis susceptibility,¹⁴ and now we evaluated the role played by bone marrow-derived cells through bone marrow
transplantation. Monocyte-derived foam cells are the principal cellular component of atherosclerotic lesions, and these cells also produce cytokines and growth factors that may affect the development and progression of atherosclerosis. As the H2 haplotype of C3H.apoE−/− mice is H2k and that of B6.apoE−/− mice is H2b, we constructed C3.SW.apoE−/− mice, which have the same H2b haplotype as B6.apoE−/− mice, for transplantation. C3.SW.apoE−/− and C3H.apoE−/− mice are genetically identical except for the H2 region. The present finding that B6.apoE−/− mice reconstituted with bone marrow of C3.SW.apoE−/− mice developed similar lesion size as those reconstituted with syngeneic bone marrow indicates that macrophages or other marrow-derived cells contribute little to the difference in atherosclerosis susceptibility. Nevertheless, the finding that C3.SW.apoE−/− mice reconstituted with bone marrow of B6.apoE−/− mice developed smaller aortic lesions than those reconstituted with syngeneic bone marrow suggest that B6 marrow-derived cells actually protect against atherosclerosis.

The huge impact of H2b haplotype on the development of atherosclerosis in C3.SW.apoE−/− mice after exposure to radiation was not expected. H2, which was named for its role in histocompatibility more than 60 years ago by George Snell, is orthologous with the human HLA (human leukocyte antigen) or MHC. The MHC region is located on chromosome 17 in the mouse and on the short arm of chromosome 6, 6p21.3, in man. This region has been shown to be linked to radiation-induced pulmonary fibrosis in the mouse. As pulmonary fibrosis and atherosclerosis are chronic inflammatory diseases, it is highly likely that both diseases are affected by the same genes causing cytokine-driven inflammation and tissue destruction.

C3.SW.apoE−/− mice reconstituted with bone marrow from either strain developed much larger atherosclerotic lesions than B6.apoE−/− counterparts despite the fact that they share the same H2b haplotype. The reason for this is unknown. Previous studies of F2 mice derived from B6.apoE−/− and C3H.apoE−/− mice showed that multiple loci contribute to atherosclerotic lesion
formation and alleles derived from C3H may contribute to the susceptibility to atherosclerosis while alleles derived from B6 may contribute to the resistance to atherosclerosis. Thus, it is likely that genes outside the H2 region protect against radiation-enhanced atherosclerosis in B6.apoE−/− mice. The present observation that C3.SW.apoE−/− mice reconstituted with B6 bone marrow developed smaller atherosclerotic lesions than those reconstituted with C3.SW bone marrow supports this speculation. In addition, C3.SW.apoE−/− mice had higher non-HDL cholesterol levels than B6.apoE−/− mice, which could be partially responsible for the increased lesion formation.

Sublethal irradiation alone resulted in accelerated formation of atherosclerotic lesions in C3.SW.apoE−/− mice without leading to significant alterations of plasma lipid levels. However, the effect was much smaller when lesion size was compared to that of those mice that had experienced lethal irradiation and bone marrow transplantation. This discrepant result may reflect the dose-dependent effect of radiation on atherosclerosis rather than an effect inherited from the transplantation.

The present finding that wild-type C3.SW mice were totally resistant to diet-induced atherosclerosis is in agreement with previous observations made in C3H/HeJ mice. However, C3.SW and C3H/HeJ mice that received lethal irradiation and bone marrow transplantation developed atherosclerosis on the high fat diet. This result is consistent with previous conclusions that irradiation results in accelerated atherosclerosis in wild-type B6 mice or rabbits fed an atherogenic diet. When compared to C3H/HeJ mice receiving syngeneic marrow transplantation, C3.SW mice displayed 5- to 6-fold increases in atherosclerotic lesion size, indicating that genes within the H2 region also promote diet-induced atherosclerosis following irradiation.

ApoE deficiency in bone marrow-derived cells has been shown to markedly increase
susceptibility of B6 mice to atherosclerosis in the absence of a significant influence on plasma lipid levels.\textsuperscript{33, 34} In contrast, we found that reconstitution with apoE\textsuperscript{−/−} bone marrow had little influence on the susceptibility of C3.SW or C3H/HeJ mice to atherosclerosis. This finding is consistent with our previous observation of C3H/HeJ mice.\textsuperscript{19} It is also highly unlikely that the absence of apoE bone marrow-derived cells was responsible for radiation-enhanced atherosclerosis in C3.SW.aPOE\textsuperscript{−/−} mice.

In summary, we have demonstrated for the first time that the MHC is a major genetic determinant of susceptibility to radiation-induced atherosclerosis in mice. Since the MHC is one of the most clearly defined genetic determinants affecting almost every autoimmune disease and some inflammatory diseases, further investigations of susceptibility genes with this radiation hypersensitivity model may uncover critical common pathways shared by these conditions.

**Funding Sources:** This work was supported by National Institutes of Health grant HL82881.

**Conflict of Interest Disclosures:** None.

**References:**


**Figure Legends:**

**Figure 1** - Atherosclerotic lesions in the aortic root of female B6.apoE<sup>−/−</sup> and C3.SW.apoE<sup>−/−</sup> mice transplanted after lethal irradiation with donor bone marrow from either B6.apoE<sup>−/−</sup> or C3.SW.apoE<sup>−/−</sup> mice. Four groups of experiments were performed: C3.SW.apoE<sup>−/−</sup>→B6.apoE<sup>−/−</sup> (n=16), B6.apoE<sup>−/−</sup>→B6.apoE<sup>−/−</sup> (n=14), B6.apoE<sup>−/−</sup>→C3.SW.apoE<sup>−/−</sup> (n=23), and C3.SW.apoE<sup>−/−</sup>→C3.SW.apoE<sup>−/−</sup> (n=20). After transplantation, mice were fed a chow diet for 16 weeks. Values are expressed as means ± SE. * P < 0.05 vs. B6.apoE<sup>−/−</sup> recipients.

**Figure 2** - Plasma cholesterol and triglyceride levels in female B6.apoE<sup>−/−</sup> (n=14) and
C3.SW.apoE−/− mice (n=20) reconstituted with syngeneic bone marrow. Blood was collected from overnight fasted mice at 16 weeks after transplantation. Values are mean ± SEM. * P < 0.05 vs. B6.apoE−/− mice.

**Figure 3** - Comparison between female C3.SW.apoE−/− (n=20) and C3H.apoE−/− mice (n=12) reconstituted with syngeneic bone marrow in atherosclerotic lesion size at 16 weeks after transplantation. Values are expressed as means ± SE. * P < 0.05 vs. C3H.apoE−/− mice.

**Figure 4** - Atherosclerotic lesion size in the aortic root of female C3.SW.apoE−/− mice with (n=10) or without irradiation (n=5). Mice were irradiated with a single dose of 0 or 600 rads. After irradiation, mice were fed the chow diet for 16 weeks. Values are means ± SE. * P < 0.05 vs. non-irradiated mice.

**Figure 5** - Plasma cholesterol and triglyceride levels in female C3.SW.apoE−/− mice with (n=9) or without irradiation (n=10). Values are means ± SE. Radiation with a single dose of 600 rads had no significant influence on plasma lipid levels of the mice.

**Figure 6** - Atherosclerotic lesion size in the aortic root of wild-type female C3.SW or C3H mice. Mice were lethally irradiated and transplanted with bone marrow from apoE−/− or apoE+/+ mice. Four weeks after transplantation, mice were started on an atherogenic diet and maintained on the diet for 12 weeks. One group of C3.SW mice did not receive irradiation and transplantation but were challenged with the atherogenic diet. Values are means ± SE. * P < 0.05 vs. C3H mice. C3.SW (n=9): wild-type C3.SW mice receiving no irradiation and no transplantation; C3.SW→C3.SW (n=9): lethally irradiated wild-type C3.SW mice were reconstituted with bone marrow from wild-type C3.SW mice; C3.apoE−/−→C3.SW (n=15): lethally irradiated wild-type C3.SW mice reconstituted with bone marrow from C3.SW.apoE−/− mice; C3H→C3H (n=15): lethally irradiated wild-type C3H/HeJ mice reconstituted with bone marrow from wild-type C3H/HeJ mice; C3H.apoE−/−→C3H (n=14): lethally irradiated wild-type C3H/HeJ mice were reconstituted with marrow from C3H.apoE−/− mice.
Atherosclerotic lesion (μm²/section x 1000)

C3.SW.aPOE-/-.  B6.aPOE-/-.  B6.aPOE-/-.  C3.SW.aPOE-/-.
Plasma lipid levels (mg/dl)

- Total cholesterol
- HDL
- Triglyceride

C3H.SW.apoE-/-
B6.apoE-/-
Atherosclerotic lesion (μm²/section x 1000)

Non-irradiated

Irradiated

*
Plasma lipid levels (mg/dl)

- Total cholesterol
- HDL
- Triglyceride

Non-irradiated
Radiated
Atherosclerotic lesion
(μm²/section x 1000)

C3.SW

C3.SW

C3.SW

C3.aapoE

C3.H

C3.H

C3.aapoE

C3.H

C3.H

C3.H

C3.H

C3.H

C3.H

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Circ Cardiovasc Genet. published online August 20, 2010;
Circulation: Cardiovascular Genetics is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1942-325X. Online ISSN: 1942-3268

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