Microvascular alterations leading to disruptions in the blood supply contribute to both the therapeutic and the pathophysiological effects of ionizing radiation in exposed tissues. Although the larger vessels are relatively insensitive, spontaneous rupture and atherosclerotic vascular disease are associated with therapeutic irradiation for cancers of the head, neck, and chest. With the exception of arterial localization and age of onset, radiation-associated atherosclerosis is similar to idiopathic atherosclerosis. Shared histological findings include accumulations of macrophage-derived foam cells, intimal thickening, fibrosis, elastic degeneration, and calcification. Studies in animals have confirmed that atherosclerosis is a direct effect of radiation. Doses similar to or lower than those used in fractionated radiotherapy promote marked diffuse lesions within the radiation port. In both humans and animal models, the atherogenic effects of radiation appear to be more extensive in the presence of hypercholesterolemia, an observation that could reflect mechanistic similarities with atherosclerosis occurring in the absence of radiation. A number of acute effects, including endothelial damage, lipid and inflammatory cell infiltration, and lysosomal activation, have been described, but the primary mechanisms by which radiation promotes atherosclerosis have not been identified.

Recent evidence that reactive oxygen species (ROS) are involved in atherogenesis (eg, see Reference 14) provides an obvious connection with radiation. Radiolytic hydrolysis (H₂O → H₂O + e⁻) leads to the formation of a number of ROS, including the superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and the hydroxyl radical (HO•), with O₂⁻ being a major product when O₂ is present. A key pathophysiological role for O₂⁻ (or its reactive by-products) has been suggested on the basis of observations that superoxide dismutase (SOD) inhibits radiation-induced changes in a number of biological end points, including enzyme activity, membrane integrity, DNA damage, cell transformation, and cell and organism survival. SOD also affects a number of events putatively involved in atherogenesis, including cell-mediated lipoprotein oxidation and leukocyte adhesion to the vascular endothelium, thereby implicating a key role for O₂⁻ in this condition as well.

In view of the proposed involvement of O₂⁻ in the pathophysiological effects of both radiation and atherogenesis, we hypothesized that increased O₂⁻ formation contributes to radiation-induced atherosclerosis. Our objectives in the current studies were to examine the effects of upper thoracic exposure to ionizing radiation on atherosclerotic lesion formation in the C57BL/6 mouse model and to determine whether any such effects differ in mice overexpressing CuZn-SOD, the major intracellular SOD isoenzyme.
Methods

Animals
Studies were performed using atherosclerosis-susceptible C57BL/6J mice obtained from Jackson Laboratory (Bar Harbor, ME) and the transgenic strain C57BL/6-TgN(SOD1)10cje (with nontransgenic littermates as controls, which were bred in our laboratory). The latter strain possesses a 14-kb segment encompassing the entire human CuZn-SOD gene (in 8 tandem copies) and all necessary regulatory sequences within the C57BL/6 background.26 Transgenic mice were identified using polyacrylamide gel electrophoresis separation of red blood cell extracts with staining for SOD activity as previously described.27 Owing to sex differences in atherosclerosis susceptibility, only female mice were used. Animal care and handling procedures were in accordance with AAALAC regulations and experimental protocols were approved by the LBNL Animal Welfare Research Committee.

Radiation Exposures and Dietary Conditions
Radiation exposures were performed on mice between 10 and 14 weeks of age. After anesthetization with intraperitoneal Avertin, the animals were placed in small Plexiglas holders. Radiation was delivered in a single dose (2 to 8 Gy; 200 to 800 rad) with a dose rate of 0.35 Gy/min by using a Philips RT250-keV x-irradiator. In most experiments, the head, neck, and lower abdominal area were protected by placing lead shielding on the exterior of the animal compartments. All experiments employed sham-irradiated controls.

In addition to radiation dose, experimental variables included standard chow versus the high-fat diet (15% fat, 1.25% cholesterol, 0.5% sodium cholate),28 and time between radiation exposure and initiation of the high-fat diet (0, 7, or 14 days). The use of fat-fed as opposed to genetically altered mice (eg, apoE-knockouts) to investigate the effects of an atherogenic lipoprotein profile was preferred, because lipoprotein levels were not elevated before or during the radiation exposure.

Atherosclerosis Measurements
Aortic lesion areas were determined by quantitative lipid staining of serial sections of the proximal aortas as previously described in detail by Paigen et al.28 In brief, the heart and upper section of the aorta were removed from the chest cavity and placed in 0.9% saline at room temperature for 1 hour, during which the blood was flushed out and the heart muscle relaxed. Hearts were trimmed of excess tissue and lipoproteins and lucigenin chemiluminescence were evaluated under refrigeration (4°C). Plasma cholesterol concentrations were measured by monitoring lucigenin chemiluminescence in the presence of identical quantities of xanthine and xanthine oxidase, as described by Ohara et al.31

Plasma Total and HDL Cholesterol Determinations
Immediately before excision of the heart (as described above), blood was removed from the chest cavity with a Pasteur pipette flushed with EDTA solution and placed in a 1.5-ml Eppendorf tube containing EDTA. Plasma was isolated by centrifugation at 2000×g under refrigeration (4°C). Plasma cholesterol concentrations were assayed using an enzymatic cholesterol kit (Boehringer Mannheim); HDL cholesterol concentrations were measured after precipitating VLDL, IDL, and LDL with polyethylene glycol.29

Lucigenin Chemiluminescence
Aortic O2•− concentrations were measured by monitoring lucigenin-enhanced chemiluminescence, which is sensitive to nanomolar concentrations of O2•− but is unaffected by H2O2 or HO•. For these experiments, the entire upper abdominal area was irradiated, and measurements were performed using the abdominal aorta from the aortic arch to above the renal artery. The aorta was excised from anesthetized animals after perfusion with PBS containing 1 mmol/L EDTA and removal of the adventitia. The excised aorta was cut longitudinally and prepared for measurement essentially as described by Brandes and Mugge.28 Chemiluminescence was monitored for a 5-minute period after dark adaptation by using a scintillation counter in the out-of-coincidence mode. After subtraction of background counts obtained with aorta-free preparations, values were normalized on the basis of tissue protein and were converted to nmol O2•− by comparing ferricytochrome c reduction and lucigenin chemiluminescence in the presence of identical quantities of xanthine and xanthine oxidase, as described by Ohara et al.31

Statistical Analyses
All values represent the mean±SE. Radiation dose effects were evaluated by repeated-measures ANOVA. Differences in aortic lesion areas between irradiated and sham-irradiated and between transgenic and nontransgenic mice were evaluated using the Mann-Whitney U test (for unequal variances). Differences in plasma lipids and lipoproteins and lucigenin chemiluminescence were evaluated using 2-sample t tests. All significance levels were derived using 2-tailed tests with P<0.05 considered as significant.

Results

Atherogenic Effects of Ionizing Radiation in C57BL/6 Mice
To evaluate the atherogenic effects of ionizing radiation in C57BL/6 mice and to determine the radiation dose requirements for such effects, we exposed animals to a single 2-, 4-, or 8-Gy dose of 250-keV x-ray radiation to the upper thorax. These doses were selected on the basis of evidence of stimulation of putative atherogenic events in isolated vascular cells and animal models,32–35 without effects on vascular or endothelial integrity. In initial studies, we confirmed the latter by ascertaining that there were no significant changes in endothelial permeability based on Evans blue dye exclusion or uptake of 125I-LDL for up to 10 days at any of the doses used (data not shown). Immediately after radiation exposures, animals were placed on a high-fat atherogenic diet for 18 weeks, after which aortic lesion area was measured.

As shown in Figure 1, mean lesion area was increased with increasing radiation dose (P=0.02 by repeated-measures ANOVA, repeated-measures ANOVA: P<0.02). In addition, the mean lesion area was increased dose dependently with a slope of 0.9. Values represent the mean±SE for 14, 5, 5, and 14 mice (from left to right). Note that values on the x axis are not continuous.
ANOVA) and was 3-fold greater in 8-Gy–irradiated than sham-irradiated mice (7800±2140 versus 2635±709 \( \mu m^2 \), respectively, \( P<0.05 \) by the Mann-Whitney U test). Thus, as previously shown in other animal models, \( 8\text{–}12 \) ionizing radiation has direct atherogenic effects in C57BL/6 mice.

The influence of diet on radiation-induced atherosclerosis was evaluated by conducting a parallel study in which mice were either irradiated with 8 Gy or sham-irradiated and then either maintained on chow or placed on the high-fat diet for 18 weeks (a \( 2\times2 \) design). As is clearly evident in Figures 2 and 3, there were no lesions in chow-fed mice regardless of radiation exposure status. These results suggest that, in the C57BL/6 mouse model, radiation alone is not sufficient to induce atherosclerosis but rather enhances the atherogenic effects of the high-fat diet. It is important to note that we did not characterize aortic cellularity or the extracellular matrix and thus cannot exclude the possibility of changes in these or other parameters in irradiated, chow-fed mice that are not reflected by lipid staining.

In numerous previous studies, the atherogenic effects of the high-fat diet in C57BL/6 mice have been linked to the more atherogenic lipoprotein profile observed under these conditions (eg, see References 36 and 37). As shown in the Table and consistent with previous reports, total plasma cholesterol concentrations were almost 3-fold higher and HDL cholesterol concentrations were \( \approx20\%\text{–}30\% \) lower in mice on the high-fat diet than the low-fat (chow) diet (\( P<0.05 \)). Radiation exposures did not exert any independent effects in fat-fed mice. HDL cholesterol concentrations were significantly lower in 8-Gy–irradiated than in sham-irradiated mice on the chow diet, but this difference did not influence atherosclerosis susceptibility, since neither of these groups developed lesions. Thus, the atherogenic effects of radiation do not involve alterations in the lipid or lipoprotein profile but rather are suggested to involve changes in the artery wall that enhance lipid deposition in the presence of a conducive lipid/lipoprotein profile.
Effects of Ionizing Radiation and CuZn-SOD

Notably, stimulation of atherogenesis occurred only when the high-fat diet was introduced within 7 days after the exposure to radiation. (See Figure 4.) When the high-fat diet was introduced 14 days after radiation exposure, the mean lesion area was the same as that in the corresponding nonirradiated, high-fat–fed group. Thus, the atherogenic effects of radiation appear to be particularly pronounced immediately after the radiation exposure.

Effects of CuZn-SOD Overexpression on Radiation-Induced Lesion Formation

Ionizing radiation is a source of ROS, including O$_2^-$, and these species have been proposed to mediate many of the deleterious effects (see References 16 through 20). To evaluate whether O$_2^-$ or its reactive by-products might be involved in mediating radiation-enhanced atherosclerosis, experiments were performed on C57BL/6 mice expressing human CuZn-SOD. We previously showed that these mice exhibit 2- to 3-fold higher SOD activities in the heart, aortic tissue, and peritoneal macrophages relative to their littermate controls. Transgenic mice were irradiated with 8 Gy and then immediately placed on the high-fat diet for 18 weeks. As shown in Figure 5 (left), the mean aortic lesion area was 2-fold lower in irradiated, high-fat–fed transgenics than in their irradiated, high-fat–fed nontransgenic littermates (3026±1590 versus 6102±1834 μm$^2$, respectively, P<0.05). This did not involve effects on plasma total cholesterol or HDL cholesterol concentrations (data not shown). Importantly, as we previously reported, SOD overexpression did not influence diet-induced atherosclerosis in the absence of radiation (see Figure 5, right). Mean lesion areas were considerably lower under these circumstances and did not differ between SOD overexpressers and their littermate controls.

Effects of Ionizing Radiation and CuZn-SOD Overexpression on Aortic O$_2^-$ Concentrations

The inhibitory effects of SOD implicate O$_2^-$ and its reactive by-products as mitigating factors in radiation-enhanced atherosclerosis. To evaluate the effects of radiation and SOD overexpression on aortic O$_2^-$ concentrations, we measured lucigenin chemiluminescence in abdominal aortas excised at various times (from 1 to 144 hours) after 8-Gy irradiation of the upper abdominal area. As shown in Figure 6, a biphasic response was observed. The first phase was characterized by an acute increase relative to baseline that was maximal at 1 hour but was no longer apparent at 8 hours. Values were ≈20% lower in SOD transgenics at both 1 and 2 hours, but these differences were not significant. The second phase was characterized by a slower, more modest increase in chemiluminescence that peaked at 72 hours in nontransgenics, when values were 50% (albeit insignificantly) higher than those observed at baseline. In contrast to the acute phase, second-phase values were 2- to 3-fold lower in SOD transgenics (P<0.05). This difference was observed from 24 to 96 hours but was no longer apparent at 144 hours. Thus, as with diet responsivity, increased lucigenin chemiluminescence and the inhibitory effects of SOD on this measure are transient responses to ionizing radiation.

Discussion

Ionizing radiation is known to predispose to the development of atherosclerosis, although the mechanisms for this effect have not been determined. Using the C57BL/6 mouse model, we have shown that radiation enhances the atherogenic effects of a high-fat diet via CuZn-SOD–inhibitable processes. Based on the transient increase in diet responsivity, the relevant effects appear to be particularly pronounced during the first week after exposure. We thus propose that radiation promotes short-lived changes in oxidative stress conditions (O$_2^-$ concentrations) in the artery wall and that atherogenic lipoproteins, which are elevated in response to the high-fat diet, must be available during this period to participate in the initiation of the disease process.
In previous studies, we showed that CuZn-SOD overexpression does not inhibit atherosclerosis in fat-fed mice in the absence of radiation, suggesting that O$_2^-$-dependent processes do not predominate in promoting atherogenesis in the nonirradiated model. Ionizing radiation is thus proposed to be a useful experimental tool for investigating atherogenic events stimulated by oxidative processes and for testing the antiatherogenic properties of antioxidants. As a means of initiating oxidative stress, radiation is preferable to many other approaches because it is noninvasive, yet it can be targeted to specific regions of the body and it can be delivered in precise doses. Another potentially useful property of radiation is its ability to affect both the intracellular and extracellular environments. A disadvantage of radiation is its direct effects on biological macromolecules such as DNA, which can independently promote disease. However, our results with CuZn-SOD transgenic mice suggest that it may be possible to discern specific atherogenic events linked to oxidative perturbations by combining radiation exposures with genetic or other manipulations affecting oxidative stress or antioxidative conditions.

The effects of radiation and SOD overexpression on aortic oxidative stress conditions were evaluated by measuring lucigenin-enhanced chemiluminescence, a sensitive indicator of O$_2^-$, at various times after radiation. We observed a biphasic response, characterized by an acute increase that was relatively insensitive to SOD overexpression, followed by a slower phase that appeared to peak at 72 hours and was markedly inhibited in SOD transgenics. The first phase is attributed to oxidative stress conditions initiated by the deposition of radiation energy. The basis of the second phase is currently unknown but could reflect the tissue response to radiation, possibly including the recruitment and activation of inflammatory cells. The marked inhibitory effect of SOD overexpression on the second phase of chemiluminescence suggests that O$_2^-$-mediated events occurring during this period should be investigated for their role in promoting the transient, SOD-inhibitable atherogenic effects of radiation.

Although the current study did not reveal specific mechanisms underlying radiation-enhanced atherosclerosis and the inhibitory effects of SOD, several key processes are proposed based on the known effects of radiation, current models of atherogenesis, and the proposed effects of O$_2^-$. Chief among these is lipoprotein oxidation, which is now generally believed to play a key role in atherogenesis. Radiation-induced changes in aortic oxidative stress could lead to increased oxidation of lipoproteins, which then could mediate a multitude of atherogenic effects. This sequence of events could explain the critical role of the high-fat diet and the requirement that the diet be introduced soon after the radiation exposure (ie, when oxidant stress is increased), as well as the inhibitory effects of SOD, which have been shown to inhibit inclusion of cell-mediated lipoprotein oxidation in vitro.

One of the key atherogenic effects of oxidized lipoproteins is the recruitment of inflammatory cells, which secrete a number of atherogenic signaling molecules, increase the local oxidative burden, and serve as progenitors of the lipid-laden foam cells that form the basis of the fatty streak lesion. Lipid oxidation by-products present in oxidized lipoproteins have been shown to induce the expression of leukocyte adhesion molecules and to alter the chemotactic behavior of monocytes/macrophages in a manner expected to promote their retention in the artery wall. Although this sequence of events is consistent with the lipoprotein oxidative modification hypothesis, it is not necessary to invoke a role for lipoproteins in inducing inflammation in irradiated tissues. Radiation has been shown to promote rapid induction of adhesion molecules, including E-selectin and intercellular adhesion molecule-1, within 3 to 6 hours in isolated vascular endothelial cells at doses as low as 1 and 5 Gy, respectively. Similar effects have been demonstrated in the lung of irradiated mice at doses as low as 2 Gy. These responses appear to be linked to activation of the oxidant-responsive regulatory element nuclear factor-kB. Radiation could thus directly promote inflammation, with lipoproteins serving in a secondary role to enhance and/or perpetuate this response.

In summary, we have presented evidence that ionizing radiation promotes transient vascular alterations that enhance atherogenesis in fat-fed mice and that these effects are inhibited by CuZn-SOD overexpression. On the basis of these results and current models of atherogenesis, we have proposed that the atherogenic effects of radiation may involve ROS-mediated promotion of lipoprotein oxidation and vascular inflammation. Studies addressing these issues are currently under way.

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CuZn-SOD Inhibits Radiation-Induced Atherosclerosis


Ionizing Radiation Accelerates Aortic Lesion Formation in Fat-Fed Mice via SOD-Inhibitable Processes
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