Ionizing Radiation Accelerates the Development of Atherosclerotic Lesions in ApoE^{-/-} Mice and Predisposes to an Inflammatory Plaque Phenotype Prone to Hemorrhage

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After radiotherapy treatment, there is an increased incidence of localized atherosclerosis in patients with Hodgkin's disease, breast cancer, and head and neck cancer. Here, we established a mouse model to study the development and progression of radiation-induced atherosclerosis and to compare the phenotype of these lesions with age-related atherosclerosis. Atherosclerosis-prone ApoE^{-/-} mice fed a regular chow diet received single radiation doses of 14 Gy or sham treatments (0 Gy) to the neck, including both carotid arteries. At 22, 28, and 34 weeks after irradiation, blood samples were taken, and the arterial tree was removed for histological examination. Cholesterol levels in irradiated mice were not significantly different from age-matched controls, and markers of systemic inflammation (soluble intercellular adhesion molecule-1, soluble vascular cell adhesion molecule-1, and C-reactive protein) were not elevated. The lesions in irradiated arteries were macrophage rich, with a remarkable influx of inflammatory cells, predominantly granulocytes. Intraplaque hemorrhage and erythrocyte-containing macrophages were seen only in lesions of irradiated arteries. Based on these data, we propose that irradiation accelerates the development of macrophage-rich, inflammatory atherosclerotic lesions prone to intraplaque hemorrhage. (*Am J Pathol* 2006, 168:649–658; DOI: 10.2353/ajpath.2006.050409)

Earlier diagnosis and better treatment options have led to improvements in cancer-specific survival for most tumor types, but this also results in an increased number of patients at risk for developing treatment-related side effects. At least one-half of all cancer survivors will have received radiotherapy during their treatment, and vascular injury is the major cause of late radiation morbidity.

A meta-analysis of radiotherapy trials for women with early breast cancer showed that the benefit of radiotherapy (5% reduction in cancer related deaths) was offset by a 4% increase in non-breast-cancer-related mortality, mainly late vascular disease that became apparent with longer follow-up.¹ Radiotherapy for Hodgkin's disease also results in increased cardiovascular disease.^{2–4} A cohort study of >1200 Dutch patients demonstrated a 6.3 relative risk (RR) of developing cardiovascular disease for the whole group, and patients irradiated at age <21 years had even higher risk (RR, 13.6).⁴ Retrospective studies in head and neck cancer patients have identified stenosis and reduced blood flow or increased intima-media thickness of the irradiated section of the carotid artery.^{5–9} A cohort study of 367 head and neck cancer patients irradiated at the Netherlands Cancer Institute also showed a significantly increased risk of ischemic stroke in a relatively young population that would not normally be associated with atherosclerosis.¹⁰ The overall RR of stroke in the patient cohort was 5.6 times that expected, and this increased to 10.1 after a follow-up of >10 years.

There is, therefore, good evidence to identify radiation as an independent risk factor in human vascular disease, and this is supported by experimental studies in hypercholesterolemic animals.^{11–15} However, the mechanisms involved are not fully understood, and it is not known

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Table 1. ApoE^{-/-} Mice Included in Analysis of Atherosclerotic Lesions

Sex/follow-up	At treatment		At sacrifice		N
	Age (weeks)	Weight (g)	Age (weeks)	Weight (g)	
Females/22 weeks					
0 Gy	13.9 ± 0.3	20.5 ± 0.5	35.9 ± 0.3	24.2 ± 0.6	10
14 Gy	14.2 ± 0.2	20.8 ± 0.3	35.6 ± 0.7	21.5 ± 0.3	14
<i>P</i> value		ns		0.001	
Males/22 weeks					
0 Gy	9.6 ± 0.2	25.3 ± 1.1	31.6 ± 0.2	29.4 ± 0.7	7
14 Gy	9.9 ± 0.3	25.0 ± 0.5	31.9 ± 0.3	27.6 ± 0.4	12
<i>P</i> value	ns		ns		
Males/28 weeks					
0 Gy	10.0 ± 0.3	20.1 ± 0.6	38.0 ± 0.3	28.9 ± 1.0	7
14 Gy	9.7 ± 0.3	23.8 ± 1.0	37.9 ± 0.3	28.5 ± 0.7	7
<i>P</i> value		ns		ns	
Males/34 weeks					
0 Gy	10.4 ± 0.9	21.8 ± 0.7	43.7 ± 1.1	28.9 ± 0.8	7
14 Gy	9.7	23.0 ± 0.6	43.1 ± 0.7	25.5 ± 1.3	7
<i>P</i> value		ns		0.05	

Group means ± SEM. *P* values compare 0- and 14-Gy groups. ns = *P* > 0.05.

whether this process represents the same etiology as age-related atherosclerosis. A review of clinical records from United States Army personnel irradiated for mediastinal malignancies concluded that radiation-induced intimal plaque was similar to classical atherosclerotic coronary disease, although medial thinning and adventitial fibrosis were more prominent after irradiation.¹⁶ Several anecdotal reports identify fibrosis and ischemia of irradiated vessel walls, rather than accumulation of lipid-filled foam cells, but this may well reflect the presence of end-stage atherosclerosis and healed ruptured plaques. Systematic data on composition and stability of early radiation-induced atherosclerosis and its progression are lacking.

With increasing attention on quality of life aspects of cancer treatments, more knowledge is required on the mechanisms of development and management of late side effects. Specific interventional treatments to reduce the severity of radiation-induced vascular injury would have significant clinical impact in cancer therapy, both in terms of quality of life and overall survival. A suitable animal model mimicking the development of radiation-induced atherosclerosis in irradiated cancer patients would allow us to study the mechanisms of initiation and progression of these lesions and to evaluate candidate intervention drugs. Mice lacking functional ApoE (the main ligand recognized by the murine low density lipoprotein receptor, LDLR) have elevated plasma cholesterol levels and develop arterial lesions with age, unlike wild-type mice. These mice have been extensively used to study the development of age-related atherosclerosis in the absence of radiation.¹⁷ The goal of the present study was therefore to characterize radiation-induced atherosclerotic lesions in ApoE^{-/-} mice, with specific reference to the inflammatory component and the presence of intraplaque hemorrhage, and to compare these with lesions in age-matched nonirradiated mice.

Materials and Methods

Mice

ApoE^{-/-} mice on a C57Bl/6J background were bred in isolator cages at the breeding facility of the Netherlands Cancer Institute. From the time of weaning, mice were housed in filter-top cages and given free access to standardized mouse chow (4% fat; catalog no. 4064.01; Arie Blok Diervoeding, Woerden, The Netherlands) and acidified drinking water. At a mean age of 10 weeks (males) or 14 weeks (females) and a body weight of 20 to 25 g, age- and sex-matched mice were randomly allocated to receive irradiation or sham treatment (Table 1). It was important that mice were the correct weight at the time of treatment to ensure correct positioning of the carotid arteries within the irradiation field. A total of 24 female and 47 male ApoE^{-/-} mice were included in the quantitative analyses of atherosclerotic lesions at 22 to 34 weeks after treatment. A small number of animals (six controls and four irradiated) were also qualitatively evaluated at 13 weeks after treatment. Experiments were performed in accordance with the national regulations for animal experimentation, and the local animal welfare committee approved all experimental protocols.

Irradiation Procedure

Mice were irradiated with single doses of 14 or 0 Gy (sham treatment) to the neck region. During irradiation or sham treatment, the mice were immobilized in the prone position in acrylic plastic (Perspex) jigs with the non-target areas shielded with lead. Irradiation was in a dorso-ventral direction, with 250-kV X-rays, operating at 12 mA and filtered with 0.6 mm of copper. The field size was 20 × 15 mm, encompassing both carotid arteries, the aortic arch, and apical portion of the heart but with the lung outside the 100% isodose region (Figure 1). Low-dose, scattered irradiation (maximum 10%) was deliv-

Figure 1. Irradiation field and dose distribution to arterial tree. **A:** The irradiation field of 2.0×1.5 cm delivered 100% of prescribed dose to both carotid arteries (including the bifurcation), the aortic arch, and depending on exact positioning of the mouse, the apical portion of the heart. The mid section of the heart received about 8 to 10% of full dose, and the majority of the lungs received <4% scattered radiation dose. **B:** Photograph of the excised arterial tree (with heart and kidneys) in relation to the schematic irradiation field.

ered to the apical regions of thoracic structures as shown. Groups of age- and sex-matched mice were sacrificed at 22, 28, or 34 weeks after irradiation (Table 1), and arterial lesions, blood cholesterol levels, and systemic markers of inflammation were quantitatively analyzed.

Blood Sampling and Analysis

At the pre-planned analysis times, mice were euthanized after overnight fasting, and blood samples (0.5 to 0.9 ml per mouse) were taken by cardiac aspiration. Aliquots of whole blood were collected in EDTA and centrifuged at 3500 rpm for 15 minutes at 4°C. Plasma samples were frozen at -70°C for subsequent analysis of cholesterol levels and levels of the systemic inflammatory markers soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), and high sensitivity C-reactive protein (CRP). Plasma cholesterol and triglyceride levels were determined by standard colorimetric enzyme assays (Cobas Integra 400; Roche Diagnostics, Almere, The Netherlands). Plasma levels of sVCAM-1, sICAM-1, and CRP were measured according to manufacturer's instructions with enzyme-linked immunosorbent assay kits from R&D Systems (Abingdon, UK), Endogen (Etten-Leur, The Netherlands), and Kamiya Biomedical Company (Seattle, WA), respectively. All measurements were done in duplicate, and the mean of two values from each sample was used in further analyses.

Serum levels of free thyroxine (T4) and thyroid stimulating hormone (TSH) were measured in a subgroup of four irradiated and four control mice to check for possible reduction in thyroid function after cervical irradiation. These measurements were done using an electro-chemiluminescence assay kit from Roche, according to manufacturer's instructions.

For a subset of mice, blood was collected in heparin, centrifuged at 1500 rpm for 4 minutes and used immedi-

ately for fluorescence activated cell sorter analysis of white blood cell content. The percentages of granulocytes, monocytes, and T and B lymphocytes were measured in whole blood taken from five irradiated and five sham-treated, age-matched male mice at 28 weeks after treatment. The following combinations of antibodies and dilutions were used: Mac1-phycoerythrin (PE) (1:300) with Gr1-fluorescein isothiocyanate (1:750) and 6B2-PE (1:300) with CD3-fluorescein isothiocyanate (1:200).

Tissue Handling

Immediately after euthanasia and blood sampling (cardiac aspiration), the arterial system was perfused under standard pressure (100 mm Hg) with sodium-nitropruside in phosphate-buffered saline (0.1 mg/ml, 3 minutes), followed by 1% paraformaldehyde fixative (3 minutes) before excision of the cervical, thoracic, and abdominal arterial tree and the lungs and heart. The entire preparation was attached to a cork sheet and fixed for 24 hours in 1% paraformaldehyde before transfer to 70% alcohol. The aortic arch, carotid arteries, descending thoracic aorta, and renal arteries were embedded in paraffin and 4- μm longitudinal, serial sections were cut. Lung and heart were also routinely embedded and sectioned.

Morphometric Analysis of Plaque

For the quantitative analysis of plaque area and number, approximately 35 longitudinal, serial sections were made from each carotid (all mice) and each renal artery (female mice only); these were numbered sequentially. Sections 4, 9, 14, 19, 24, 29, and 34 were stained with hematoxylin and eosin (H&E) and examined for plaque. Plaque area was measured on four of these sections per artery, selected to cover the central part of each lesion, and the average of these measurements was recorded per lesion. Total numbers and areas of lesions in left and right carotid or renal arteries were calculated per mouse, and results (group means and SEM) were reported for male and females separately. All plaques were categorized as initial lesions (macrophage rich, without a thick fibrous cap) or advanced (well-defined necrotic/lipid core or thick fibrous cap), using the modified American Heart Association criteria.¹⁸ Group mean plaque areas were only calculated when there were at least three of a particular class of lesions in the group. The relative collagen and lipid core content was determined by dividing these areas by individual plaque area. Lesions in the aortic root were evaluated for phenotype (female mice only), but plaque area was not quantified. Morphometric parameters were analyzed using a microscope coupled to a computerized morphometry system (Leica Qwin V3; Leica, The Netherlands), as previously described.¹⁹

Immunohistochemistry and Histological Staining

Macrophage and leukocyte counts were done on one of the central sections per artery, and results were expressed as the percentage of antibody-positive cells in

Table 2. Cholesterol Levels in Plasma from ApoE^{-/-} and Wild-Type C57/Bl Mice

Mice	Total cholesterol	High-density lipoproteins	Low-density lipoproteins	Triglycerides
ApoE ^{-/-} 14 Gy (n = 18)	11.5 ± 0.5	3.9 ± 0.2	6.6 ± 0.4	1.2 ± 0.1
ApoE ^{-/-} 0 Gy (n = 7)	10.6 ± 1.1	4.3 ± 0.3	5.9 ± 0.8	1.1 ± 0.2
C57/Bl 0 Gy (n = 6)	2.5 ± 0.2	2.3 ± 0.5	<0.2	0.7 ± 0.1
	P = 0.003	P = 0.003	P = 0.003	ns

Values are group mean concentrations (mmol/L) ± SEM of samples from male and female mice combined. P values compare 0-Gy ApoE^{-/-} mice with 0-Gy C57/Bl6 wild-type mice. ns = P > 0.05.

total cells counted. Mean inflammatory cell count was only calculated when there were at least three lesions per group. Sections were immunolabeled with Mac3 (1:30; Pharmingen) and CD45 (1:50; Pharmingen) to detect inflammatory cells. Sections were also examined for the presence of collagen (Sirius Red), fibrin (Martius-scarlet-blue trichome staining), erythrocyte-containing macrophages (H&E) as an indication of previous intraplaque hemorrhage, and atypical, swollen endothelial cells (ECs). These sections were scored semiquantitatively to identify the presence of these characteristics in carotid arteries of individual mice.

Statistics

Data for inflammatory markers of systemic effects and quantitative morphometric analyses of plaques were expressed as means ± SEM, and irradiated and unirradiated groups were compared using a non-parametric Mann-Whitney U-test. Data for semiquantitative assessment of plaque thrombosis or atypical ECs were expressed as incidence of carotid arteries containing lesions with the phenotype, and groups were compared using a χ^2 test. Group differences were considered statistically significant at P < 0.05.

Results

Evaluation of Systemic Effects

Irradiation of the carotid arteries induced weight losses of about 1 g during the 1st week, and irradiated mice were an average of 6% (males) to 11% (females) lighter than age-matched controls at the time of euthanasia (Table 1). Only three irradiated mice and one control mouse died

before the planned sacrifice time; the cause of death could not be determined.

Cholesterol levels of unirradiated female and male ApoE^{-/-} mice did not differ significantly; lipid data from males and females were therefore pooled for comparison with irradiated ApoE^{-/-} mice and nonirradiated wild-type C57BL/6J mice. Irradiation of carotid arteries did not significantly influence cholesterol or triglyceride levels in ApoE^{-/-} mice, but total cholesterol levels were four times higher than in wild-type mice (Table 2). This increase was mainly in the low-density lipoprotein cholesterol fraction, which was >30 times higher in ApoE^{-/-} mice.

There was no increase in systemic markers of inflammation (sICAM, sVCAM, and CRP) at 22 weeks after irradiation of carotid arteries, compared with age-matched unirradiated ApoE^{-/-} mice (Table 3). FT4 and TSH levels were also unchanged after irradiation (Table 3), indicating that thyroid function was normal. Fluorescence activated cell sorter analysis of whole blood taken at 22 weeks after treatment showed that there was no significant shift in the white cell counts after irradiation of the carotid artery, although considerable variations were seen between mice (Table 4). Histological examination of heart and lung tissue from irradiated mice did not reveal any abnormalities or generalized inflammation (data not shown). These results show that local irradiation of the carotid arteries did not influence cholesterol metabolism or thyroid function or induce systemic inflammation.

Plaque Burden in ApoE^{-/-} Mice

A pilot study with mice killed 13 weeks after treatment identified only 1.0 carotid artery lesion per animal in the controls (n = 6) and 1.25 lesions per animal in the irradiated mice (n = 4). These lesions were not quantitatively

Table 3. Plasma or Serum Concentrations of Systemic Markers of Inflammation and Thyroid Function in Irradiated and Age-Matched ApoE^{-/-} Mice

		0 Gy	N	14 Gy	N	P value
		Mean (SEM)		Mean (SEM)		
sICAM (μ g/ml)	M ¹	18.1 (1.0)	7	17.4 (0.4)	9	ns
	F ¹	23.2 (0.5)	10	25.1 (1.2)	13	ns
sVCAM (ng/ml)	M ¹	845 (60)	6	805 (43)	9	ns
	F ¹	820 (84)	9	724 (137)	14	ns
CRP (ng/ml)	M ¹	<75	6	<75	9	ns
	F ¹	<75	10	<75	11	ns
TSH (μ IU/ml)	M ²	0.018 (0.003)	4	0.016 (0.003)	4	ns
FT4 (ng/dl)	M ²	1.58 (0.07)	4	1.71 (0.05)	4	ns

M¹, plasma from male mice 22 weeks after treatment; F¹, plasma from female mice 22 weeks after treatment; M², serum from male mice 28 weeks after treatment; ns, not significant (P > 0.05).

Table 4. FACS Analysis of White Blood Cell Distributions in Irradiated and Nonirradiated Male ApoE^{-/-} Mice at 28 Weeks after Treatment

Cell type	0 Gy	14 Gy	P value
Monocytes	18.5 ± 7.5	10.4 ± 0.9	ns
Granulocytes	32.6 ± 5.0	21.6 ± 4.8	ns
B cells	36.3 ± 3.5	39.9 ± 6.6	ns
T cells	11.6 ± 2.6	14.4 ± 3.4	ns

Values are means ± SEM of five mice per group. ns, not significant, $P > 0.05$.

analyzed due to low numbers. From 22 to 34 weeks after treatment, the mean number of lesions in carotid arteries of irradiated mice was greater than in age- and sex-matched controls (Figure 2 C). At 22 weeks, the vast majority of plaques in the irradiated arteries were classified as initial lesions (85 and 96% of total in female and males, respectively), but at later times, only 40% of the lesions in irradiated carotids were classified as initial (Figure 2A). There was no increase in the number or, with the exception of the 28-week male group, mean size of advanced plaques after irradiation (Figure 2, B and E). The total plaque burden was also not significantly greater in irradiated mice compared with age-matched controls at individual time points (Figure 2F). However, there was a trend for an increase in total plaque in irradiated mice at later times, and if all mice at 28 and 34 weeks after treatment were considered together, there was a significant increase in total plaque burden in irradiated mice (0.68 ± 0.10 versus 0.33 ± 0.09 mm², $P = 0.01$). Wild-type C57Bl/6J mice were not irradiated in this study. However, in an evaluation of carotid arteries from non-atherogenic C3H mice (from a separate study), no

Figure 2. Morphometric analysis of lesions in carotid arteries. Bar graphs showing the mean number of initial (A), advanced (B), and total (C) carotid artery lesions per animal (both carotids). Mean individual plaque areas for initial and advanced lesions are shown in D and E, and total plaque area is shown in F. Open bars represent animals sham irradiated with 0 Gy, and closed bars represent 14 Gy to the carotid arteries. Lesions from female (F) and male (M) mice at 22, 28, and 34 weeks after treatment are indicated on the figure. Error bars show 1 SEM, and * indicates significant differences between irradiated and control groups ($P < 0.05$ by Mann-Whitney).

Figure 3. Quantitative analysis of inflammatory content of carotid artery lesions. Bar graphs showing the mean percentages of Mac3-positive cells (macrophages) (A and B), or CD45-positive cells (pan-leukocytes) (C and D) in carotid artery lesions of female (F) and male (M) mice at 22, 28, or 34 weeks after irradiation. Open bars represent animals sham treated with 0 Gy, and closed bars represent 14 Gy to the carotid arteries. Error bars show 1 SEM, and * indicates significant differences between irradiated and control groups ($P < 0.05$ by Mann-Whitney).

plaque lesions or pathological alterations at the vascular wall were seen 26 weeks after irradiation

Plaque Composition

The most marked difference between irradiated and unirradiated arteries was in the inflammatory content and plaque hemorrhage of carotid artery lesions. At 22 weeks after irradiation, 65% of the cellular content of initial lesions ($n = 43$) in female mice was macrophage positive, and a further 10% was CD45 positive, mainly granulocytes (Figure 3, A and C). These cell types were much less common in both initial ($n = 1$) and advanced ($n = 17$) lesions from nonirradiated arteries of age- and sex-matched mice (Figure 4, compare A and B). The macrophage-rich initial lesions in the irradiated arteries grew to large sizes without progressing to the classical advanced plaque phenotype, which accounts for the relatively high number of initial lesions in irradiated arteries. Inflammatory cells were entirely confined to the atherosclerotic lesions and were not seen in the media or adventitia of irradiated arteries adjacent to these lesions. Advanced plaques in irradiated arteries of female mice had significantly more CD45-positive cells (Figure 3D) and less collagen content than lesions in nonirradiated mice (44 ± 8 versus $73 \pm 6\%$; $P = 0.006$).

In male mice, there was no significant difference in the macrophage or CD45-positive content of initial plaques at 22 weeks after irradiation or sham treatment (Figure 3, A and C); but at later times, there was a significant increase in the macrophage content (28 weeks) and CD45 content (34 weeks) of advanced lesions from irradiated mice (Figures 3, B and D, and 4, C and D). These advanced lesions also had significantly less collagen content than lesions in age-matched controls (21 ± 5 versus $62 \pm 6\%$; $P < 0.001$ at 28 weeks). For all irradiated groups (male and female), there was an increase in the

Figure 4. Inflammatory c phenotype of lesions in irradiated carotid arteries. Representative photomicrographs showing H&E staining in the **left panels** and Mac3 staining (red color) in the **right panels**. Sections are shown from carotid arteries of female mice at 22 weeks after 0 Gy (**A**, advanced lesion) or 14 Gy (**B**, initial lesion), or male mice at 28 weeks after 0 Gy (**C**, advanced lesion) or 14 Gy (**D**, advanced lesion). The **arrows** in **A**, **C**, and **D** indicate necrotic cores in these advanced lesions. Photomicrographs were taken using a 40× objective, and the scale bar = 20 μm.

number of mice with granulocyte-rich carotid artery lesions (Table 5).

None of the lesions in nonirradiated mice showed evidence of intraplaque hemorrhage or fibrin deposits,

whereas this was occasionally seen in irradiated arteries at 22 weeks (and in one irradiated mouse in the pilot study at 13 weeks) and was common at 34 weeks after irradiation (Figure 5, A and B; Table 5). A semiquantita-

Table 5. Semiquantitative Assessment of Carotid Arteries with Lesions Containing Specific Inflammatory or Thrombotic Characteristics

Sex/follow-up	Fibrin positive	Erythrocyte- containing macrophages	Granulocytes	Atypical ECs
Females/22 weeks				
0 Gy	0/10	0/7	2/7	0/7
14 Gy	2/12	8/12	12/12	7/12
<i>P</i> value	ns	0.005	<0.001	0.011
Males/22 weeks				
0 Gy	0/7	0/7	0/7	0/7
14 Gy	1/12	4/12	9/12	7/12
<i>P</i> value	ns	ns	0.002	0.011
Males/ 28 weeks				
0 Gy	0/7	3/7	1/7	0/7
14 Gy	0/7	6/7	6/7	1/7
<i>P</i> value	ns	ns	0.008	ns
Males/ 34 weeks				
0 Gy	0/7	0/7	1/7	0/7
14 Gy	6/7	5/7	6/7	1/7
<i>P</i> value	0.001	0.005	0.008	ns

ns, not significant ($P > 0.05$).

tive examination of carotid arteries also revealed an increase in lesions with erythrocyte-containing macrophages (Figure 5C), which is indicative of previous hemorrhage. This was further substantiated by a Perl's

iron staining in selected sections (not shown). At early times after irradiation (22 weeks), atypical, swollen ECs (Figure 5D) were also seen in irradiated carotid arteries of both female and male mice (Table 5). Atypical ECs were

Figure 5. Thrombotic phenotype of lesions in irradiated carotid arteries. **A:** H&E staining showing the presence of fibrin in a lesion from an irradiated animal (indicated by the arrows); this was confirmed by Martius-scarlet-blue staining (**B**), in which the fibrin deposits are stained red. **C:** (H&E staining) erythrocyte-containing macrophages in a lesion of an irradiated animal. **D:** (H&E staining) an example of the atypical swollen endothelial cells covering the lesions of an irradiated animal (arrows). Photomicrographs were taken using a 40× objective, and the scale bar = 20 μm.

Figure 6. Morphometric analysis of lesions in renal arteries (out of field). Analysis of advanced plaques in renal arteries of female mice at 22 weeks after carotid artery irradiation with 0 Gy (open bars) or 14 Gy (closed bars). There are no significant differences between the two groups for mean number of lesions (A), mean individual plaque area (B), macrophage content of lesion (C), or leukocyte content of lesion (D).

not found in carotid arteries of nonirradiated mice or in "out-of-field" renal arteries of irradiated mice.

The advanced plaques in irradiated carotid arteries of female mice had larger lipid cores in comparison with nonirradiated carotid arteries (6.4 versus 3.9%; $P = 0.04$). In male mice, lipid cores were rarely found in lesions at 22 weeks after treatment, but at later times, lipid cores of lesions in irradiated carotids were slightly smaller than in age-matched controls (1.3 versus 3.3% at 28 weeks, $P = 0.03$, and 6.3 versus 8.6% at 34 weeks; ns).

All lesions found in the aortic root of female mice at 22 weeks after treatment were classified as advanced. Although lesion size was not quantified, lesions in the irradiated aortic roots had an inflammatory phenotype, similar to that seen in irradiated carotid arteries, and could easily be distinguished from nonirradiated vessels when scored blindly.

Analysis of "out-of-field" renal arteries in the female mice, showed that there were no differences with respect to number of plaques, plaque area (Figure 6, A and B), or the percentage of initial and advanced lesions (20 versus 19% and 80 versus 81%) between irradiated and sham-treated mice, respectively. There was also no difference in macrophage content or CD45-positive cells in renal plaques from irradiated or nonirradiated mice (Figures 6, C and D), and no difference in the size of the lipid cores was seen (7.6 ± 1.5 versus $6.6 \pm 1.6\%$, ns).

Discussion

Here, we describe a murine model of radiation-induced atherosclerosis that should be useful in understanding the pathogenic changes seen in irradiated cancer patients and provide a convenient pre-clinical tool for testing intervention strategies. High single doses of radiation were used in this study, which are not directly relevant to clinical radiotherapy. However, important differences in the phenotype of plaques developing in irradiated mice

were identified using this model. In ongoing studies, we are investigating whether these differences are also seen after repeated daily irradiation, using clinically relevant doses of 2 Gy per fraction.

Several other studies have previously demonstrated that local irradiation of arteries of hypercholesterolemic rabbits or mice results in an accelerated formation of atherosclerotic plaque.^{11–14} These studies, however, did not characterize the cellular components of the irradiated and nonirradiated lesions. The first study to demonstrate phenotypic differences between radiation-induced and age-related atherosclerotic plaque was by Schiller and colleagues.²⁰ LDLR-deficient mice were given 10 Gy total body irradiation, followed by bone marrow reconstitution. Lesions that subsequently developed in the aortic roots of irradiated mice were macrophage rich and lipid filled, whereas lesions in nonirradiated mice were collagenous and had only minimal macrophage infiltration. The influence of radiation on lesion size was dependent on the anatomical site examined in this study. Thoracic lesions were smaller in irradiated mice, but aortic root lesions were significantly larger.²⁰ Pakala et al¹⁵ also identified a vulnerable plaque phenotype in rabbits after localized irradiation. Rabbits were fed a hypercholesterolemic diet, and the iliac arteries were denuded with a balloon catheter before exposure of one artery to intravascular radiation to 15 Gy. This procedure lead to the development of larger plaques, with an increased proportion of macrophages and metalloproteinase expression and fewer smooth muscle cells in the irradiated artery compared with the nonirradiated. This is a very similar phenotype to that described in the present study, in which we have combined the use of atherosclerosis-prone ApoE^{−/−} mice with external beam irradiation of uninjured arteries to mimic the clinical situation for head and neck cancer patients receiving radiotherapy.

The results from the present study did not identify major increases in total plaque burden in irradiated carotid arteries at 22 to 34 weeks after treatment, but the quality of the plaque was markedly influenced by irradiation. There were significant increases in the number of lesions characterized by macrophage-rich cores, low collagen content, and intraplaque hemorrhage in irradiated arteries. This finding is of particular interest in the light of recent findings of an association between plaque hemorrhage, cholesterol accumulation, and macrophage infiltration.²¹ Kolodgie et al²¹ concluded that intraplaque hemorrhage provided a powerful atherogenic stimulus and was a critical event in the induction of plaque instability and rupture in human atherosclerotic lesions.

We and others have previously shown that radiation is a potent inducer of thrombotic and inflammatory changes in ECs,^{22–25} including increased production and release of thromboxane and von Willebrand factor and decreased production of prostacyclin, thrombomodulin, and ADPase. Once initiated, thrombin signaling induces permeability changes and induces leukocyte trafficking via release of selectins and adhesion molecules.^{26–28} The increased permeability of ECs after irradiation can also lead to accumulation of lipids and initiation of atherogenic changes in the presence of hypercholesterol-

emia.¹³ In the light of these known effects of radiation on ECs, it is interesting to note that we observed a significant increase in the presence of atypical, swollen ECs and intraplaque hemorrhage in irradiated arteries. It seems plausible that radiation-induced changes in EC function, combined with radiation-induced EC death and exposure of the thrombotic elements of the underlying subendothelium,^{22–25} could lead to a chronic inflammation and favor the development of a vulnerable plaque.

Systemic inflammatory or metabolic effects of the irradiation could conceivably have contributed to the inflammatory phenotype of the plaque in irradiated mice. In head and neck cancer patients treated with radiotherapy, hypothyroidism developing many years after treatment has been shown to induce hypercholesterolemia and to contribute to increased carotid intima-media thickness, an effect that can be reversed after thyroid hormone replacement therapy.^{29,30} To exclude this possibility, we measured markers of systemic inflammation and thyroid and cholesterol metabolism, but these parameters did not differ between the irradiated and nonirradiated groups. We therefore conclude that the inflammatory characteristics of the plaque in irradiated vessels was due to the local effects of the irradiation influencing the arterial pathophysiology, mediated particularly by the endothelial cell response. This conclusion is supported by the analysis of atherosclerotic lesion in "out-of-field" renal arteries, in which there were no differences in amount or phenotype of plaques between irradiated and unirradiated mice. Even within irradiated carotid arteries, inflammatory changes were entirely restricted to the plaque. This suggests that there is some interaction between the damage caused to the endothelium by irradiation and other local changes at the initiation focus of an atherosclerotic lesion within an irradiated artery.

The increased frequency of intraplaque hemorrhage and inflammatory cells in radiation-induced atherosclerotic plaques could render them more vulnerable to rupture. This knowledge enables the development of a logical intervention strategy to prevent or ameliorate the development of atherosclerotic changes in patients after radiation therapy. The animal model described in the current manuscript can be used to evaluate the efficacy of novel drugs or drug combinations in a pre-clinical setting. Potential candidates are 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins), which not only have cholesterol lowering activity but also possess anti-inflammatory and anti-proliferative properties. The use of Atorvastatin is currently being tested in a Dutch national phase III clinical trial for patients who have received neck irradiation. A second approach that might be particularly interesting for radiation-induced vascular damage is the use of nitric oxide-donating aspirin, which, compared with aspirin administration alone, has been shown to reduce inflammation and neo-intimal thickening after vascular damage in ApoE knockout mice.³¹

In conclusion, we have developed a mouse model for radiation-induced atherosclerosis and defined a number of pathological characteristics of plaque development in the irradiated field, which differ from the "age-related" plaques in ApoE knockout mice. Although the total

plaque burden was not increased, lesions in the irradiated field contained a high proportion of macrophages and granulocytes as well as more intraplaque hemorrhage. Further investigation is needed to define the underlying molecular pathogenesis of radiation-induced atherosclerotic changes.

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