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Local heart irradiation of ApoE$^{-/-}$ mice induces microvascular and endocardial damage and accelerates coronary atherosclerosis

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ABSTRACT

Background and purpose: Radiotherapy of thoracic and chest-wall tumors increases the long-term risk of radiation-induced heart disease, like a myocardial infarct. Cancer patients commonly have additional risk factors for cardiovascular disease, such as hypercholesterolemia. The goal of this study is to define the interaction of irradiation with such cardiovascular risk factors in radiation-induced damage to the heart and coronary arteries.

Material and methods: Hypercholesterolemic and atherosclerosis-prone ApoE$^{-/-}$ mice received local heart irradiation with a single dose of 0, 2, 8 or 16 Gy. Histopathological changes, microvascular damage and functional alterations were assessed after 20 and 40 weeks.

Results: Inflammatory cells were significantly increased in the left ventricular myocardium at 20 and 40 weeks after 8 and 16 Gy. Microvascular density decreased at both follow-up time-points after 8 and 16 Gy. Remaining vessels had decreased alkaline phosphatase activity (2–16 Gy) and increased von Willebrand Factor expression (16 Gy), indicative of endothelial cell damage. The endocardium was extensively damaged after 16 Gy, with foam cell accumulations at 20 weeks, and fibrosis and protein leakage at 40 weeks. Despite an accelerated coronary atherosclerotic lesion development at 20 weeks after 16 Gy, gated SPECT and ultrasound measurements showed only minor changes in functional cardiac parameters at 20 weeks.

Conclusions: The combination of hypercholesterolemia and local cardiac irradiation induced an inflammatory response, microvascular and endocardial damage, and accelerated the development of coronary atherosclerosis. Despite these pronounced effects, cardiac function of ApoE$^{-/-}$ mice was maintained.

Improvements in cancer therapy and earlier detection and diagnosis have lead to increasing numbers of cancer survivors. Unfortunately, this also means that more patients are at risk of developing treatment-related late tissue damage and mortality. Thoracic radiotherapy, given to Hodgkin's lymphoma and breast cancer patients, is widely recognized as an independent long-term risk factor for developing heart diseases [1–4]. The pathological consequences of radiation-induced heart disease following therapeutic irradiation are pericarditis, myocardial fibrosis, coronary artery disease, valvular disorders and conduction abnormalities [5–7].

In a previous study [8], the dose and time dependence of structural and functional cardiovascular damage after thoracic irradiation were investigated in C57BL/6j mice. Inflammation, especially in the epicardium, and microvascular endothelial damage leading to vascular leakage progressed with dose (2–16 Gy) and time (20–60 weeks follow-up). However, only modest and non-progressive changes in cardiac function, detected by gated SPECT, were observed in mice surviving cardiac irradiation of 2 and 8 Gy. These data indicated that the heart was able to compensate for the structural damage. Nevertheless, 16 Gy irradiation led to excessive protein leakage in the myocardium and 38% of mice failed to maintain cardiac function at 40 weeks follow-up.

C57BL/6j mice have extremely low plasma levels of cholesterol, especially low density lipoproteins, and they are resistant to the development of atherosclerosis [9]. Cardiac damage identified after irradiation in such models therefore does not include any component of macrovascular damage as a result of accelerated atherosclerosis.

The effect of irradiation on the development of atherosclerosis has been studied in ApolipoproteinE$^{-/-}$ (ApoE$^{-/-}$) mice, which have elevated cholesterol levels and do develop age-related atherosclerosis. After local carotid artery irradiation with a single dose

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of 14 Gy or fractionated doses (20 × 2 Gy), an accelerated development of inflammatory atherosclerotic plaques was observed [9,10]. Hu et al. [11] described the distribution of atherosclerotic lesions in the coronary arteries of 60 week old ApoE$^{-/-}$ mice and found relatively few lesions (approximately 4 lesions per heart) after the second level of branching of the coronary arteries, that developed independently from valvular lesions. However, the effect of irradiation on this coronary lesion development is not known.

The aim of this study is to investigate the effect of local thoracic irradiation of hypercholesterolemic ApoE$^{-/-}$ mice on cardiac structure and function, and to compare this with previous results of irradiated wild-type C57BL/6j mice in the absence of atherosclerosis [8]. This should allow us to evaluate the contribution of macrovascular (atherosclerosis) and microvascular changes in the pathology of radiation-induced cardiac damage.

Material and methods

Mice and irradiation procedure

Male ApoE$^{-/-}$ mice (C57BL/6j background), aged 10–12 weeks (bred at The Netherlands Cancer Institute), were housed in a temperature-controlled room with 12 h light–dark cycle and received standardized mouse chow (3.7% fat, RMI (E) SQC, SDS, London, UK) and water ad libitum.

Irradiation procedure was performed as described previously [8]. Mice were randomly allocated to receive single doses of 2, 8 or 16 Gy locally to the heart (irradiation field of 10.6 × 15.0 mm, including 30% lung volume) at a dose rate of 0.94 Gy/min using 250 kV X-rays, operating at 12 mA and filtered with 0.6 mm of copper, or sham treatment (0 Gy) as a control. Mice were sacrificed 20 or 40 weeks after irradiation, and hearts and lungs were collected.

Experiments were in agreement with the Dutch law on animal experiments and welfare, and in line with the international Guide for the Care and Use of Laboratory Animals (eighth edition).

Tissue preparation and histology

The heart was perfused via the aortic arch (retro-grade) with Phosphate Buffered Saline (PBS) (frozen sections) or PBS followed by 1% paraformaldehyde (paraffin sections), under lethal sodium pentobarbital anesthesia (18 mg i.p. per mouse). Immediately after perfusion, the heart was excised, divided into three parts (base, mid and apex) and frozen on dry ice or immersed in 1% paraformaldehyde. Cross-sections were cut at the level of the mid-horizontal plane of the heart from fixed paraffin-embedded tissues (3 μm) or frozen tissues (7 μm).

Paraffin sections

Sections were immuno-labeled with anti-CD45 antibody (1:5000, Becton&Dickinson, Franklin lakes, USA) or anti-CD3 antibody (1:200, Dako, Carpinteria, USA) to determine the extent of leukocyte and T-cell infiltration, respectively. The absolute number of CD45-positive leukocytes per section was counted in the left ventricular (LV) myocardium. The number of CD3-positive T cells was counted per LV myocardial area (8 random 40× photographs). Interstitial collagen was quantified in 5 randomly selected areas of the LV myocardium based on a Sirius Red staining and results were expressed as percentage tissue positive for Sirius Red, excluding perivascular collagen, relative to myocardial area. To determine if there was a pre-mortem bleeding, a Perls'-staining was performed to detect iron. Macrophages store iron by metabolizing hemoglobin from engulfed red blood cells. An albumin staining (1:2500, Abcam, Cambridge, USA) was performed to determine myocardial deposition as a measure of vascular leakage and a Congo Red staining was used as previously described [8] to detect amyloid deposits in the myocardium.

To investigate coronary atherosclerotic plaque development, transverse sections of the complete mid-part of the heart were cut, stained every 57 μm with Hematoxylin and Eosin (H&E) and analyzed for the presence and number of coronary lesions. An average of 20 slides per heart was analyzed. Results are expressed as number of coronary lesions per mouse and mean values per group are shown. Percentage necrotic core of the coronary lesions was determined by dividing the necrotic core area by total plaque area.

Frozen sections

Sections were stained with H&E to measure the myocardial thickness. Photographs of the LV wall were taken using a 5× objective and 12 measurements per heart were performed. To detect alterations in the number of macrophages after irradiation, frozen sections were stained with anti-F4/80 antibody (1:300, AbD Serotec, Dusseldorf, Germany) and counted per LV myocardial area (8 random 40× photographs).

An anti-CD31 antibody (1:50, Becton&Dickinson, Franklin lakes, USA) was used to visualize cardiac vasculature of the mid part of the heart and to quantify microvascular density (MVD). Five random areas (40× photographs) from transverse sections of the subendocardium were photographed with a CCD 2 – Color Microscope system, including a Zeiss AxioCam color camera (Axiocam HRc, Zeiss, Göttingen, Germany). Vessels beneath a size of 1.5 or above 200 μm^2 were automatically excluded from the measurements, to ensure that only microvasculature was counted. To determine functional changes in the microvasculature, a histochemical staining with Naphtol AS-MX/DMF and fast Blue BB salt was performed to detect endothelial cell alkaline phosphatase (ALP) activity. Sections were also stained with an antibody against von Willebrand Factor (vWF) (1:4000, Abcam, Cambridge, USA) as a thrombotic marker. Photographs of whole sections stained for ALP and vWF were taken with an Aperio scanner (Scanscope-XT, Aperio technologies, Vista, USA) using a 40× objective. Analyses of the percentage myocardium positive for each marker were performed in 23 and 30 mice at 20 and 40 weeks FU respectively.

Morphometric parameters were analyzed using a computerized morphometry system (Leica Qwin V3, Leica, Rijswijk, The Netherlands).

Gene expression profiling and pathway analysis

Total RNA was isolated from frozen sections (30 slides of 30 μm) of the mid part of the heart of 17 mice at 20 weeks FU (5, 4, and 8 for respectively 0, 2, and 16 Gy) and 21 mice at 40 weeks FU (6, 7, and 8 for respectively 0, 2, and 16 Gy) using Trizol® Reagent (Invitrogen Corporation, Carlsbad, USA) according to the manufacturer's protocol. The quantity of total RNA was measured using a spectrophotometer (NanoDrop, Thermo scientific, Wilmington, USA) followed by a quality check measured by Agilent 2100 Bioanalyzer with the RNA Integrity Number (RIN) (Agilent technologies, Santa Clara, USA). Samples with a RIN above 7 were used for DNAse treatment and amplified (350 ng per sample) using Illumina Totalprep RNA Amplification kit (Ambion, Grand Island, USA). Hybridization of aRNA to Illumina Expression Bead Chips Mouse Whole Genome (WG-6 vs. 2.0) and subsequent washing, blocking and detecting were performed according to the manufacturer's protocol (Illumina, San Diego, USA). Samples were scanned on the IlluminaR BeadArray™ 500GX Reader using IlluminaR BeadScan image data acquisition software (version 2.3.0.13). MouseWG-6 vs. 2.0 BeadChip contains the full set of MouseRef-8 BeadChip probes with additional 11.603 probes from RIKEN FANTOM2, NCBI REfSeq as well from the MEEBO database.