Radiotherapy and Oncology 110 (2014) 455–460

Contents lists available at ScienceDirect

Radiotherapy and Oncology

journal homepage: www.thegreenjournal.com

Atherosclerotic lesions

# Irradiation of existing atherosclerotic lesions increased inflammation by favoring pro-inflammatory macrophages

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ARTICLE INFO

*Article history:*
Received 19 June 2013
Received in revised form 8 January 2014
Accepted 12 January 2014
Available online 11 March 2014

*Keywords:*
Radiotherapy
Atherosclerosis
Macrophages

ABSTRACT

*Background and purpose:* Recent studies have shown an increased incidence of localized atherosclerosis and subsequent cardiovascular events in cancer patients treated with thoracic radiotherapy. We previously demonstrated that irradiation accelerated the development of atherosclerosis and predisposed to an inflammatory plaque phenotype in young hypercholesterolemic *ApoE*$^{-/-}$ mice. However, as older cancer patients already have early or advanced stages of atherosclerosis at the time of radiotherapy, we investigated the effects of irradiation on the progression of existing atherosclerotic lesions *in vivo*.
*Material and methods:* *ApoE*$^{-/-}$ mice (28 weeks old) received local irradiation with 14 or 0 Gy (sham-treated) at the aortic arch and were examined after 4 and 12 weeks for atherosclerotic lesions, plaque size and phenotype. Moreover, we investigated the impact of irradiation on macrophage phenotype (pro- or anti-inflammatory) and function (efferocytotic capacity, i.e. clearance of apoptotic cells) *in vitro*.
*Results:* Irradiation of existing lesions in the aortic arch resulted in smaller, macrophage-rich plaques with intraplaque hemorrhage and increased apoptosis. In keeping with the latter, *in vitro* studies revealed augmented polarization toward pro-inflammatory macrophages after irradiation and reduced efferocytosis by anti-inflammatory macrophages. In addition, considerably more lesions in irradiated mice were enriched in pro-inflammatory macrophages.
*Conclusions:* Irradiation of existing atherosclerotic lesions led to smaller but more inflamed plaques, with increased numbers of apoptotic cells, most likely due to a shift toward pro-inflammatory macrophages in the plaque.
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Cardiovascular disease (CVD) and cancer are the two leading causes of morbidity and mortality in industrialized societies [1]. Although the number of patients diagnosed with cancer grows continuously, screening and treatment have improved, leading to increased survival rates. At least half of all long-term cancer survivors will have received radiotherapy. As a consequence, new challenges are emerging due to the development of secondary illnesses caused by radiotherapy. Survivors of Hodgkin's Lymphoma or breast cancer have higher risk of stroke and coronary heart disease [2–7]. This is partly due to vascular damage and sustained inflammation leading to atherothrombosis, decades after receiving thoracic radiotherapy. Most radiotherapy treatments for breast cancer deliver more than 40 Gy in total to the tumor bed. Although only a small part of the heart is usually exposed to this high-dose, additional research dissecting the underlying causes of radiation-induced CVD is crucial for developing specific intervention therapies.

One of the main causes of cardiovascular morbidity and mortality is atherosclerosis; it is a chronic lipid-driven inflammatory disorder of the arterial wall that can give rise to acute atherothrombotic events due to plaque erosion or rupture [8]. Whereas wild-type mice have very low cholesterol levels and are resistant to atherosclerosis, hypercholesterolemic apolipoprotein E-knockout (*ApoE*$^{-/-}$) mice have elevated levels of total cholesterol and low density lipoproteins (LDL) and develop atherosclerotic lesions spontaneously with age. As we have shown previously, local irradiation of the carotid arteries with a single dose of 14 Gy accelerated the progression of atherosclerosis in young *ApoE*$^{-/-}$ mice and predisposed to macrophage-rich, thrombotic plaques, with less collagen, all features of a rupture-prone plaque [9,10]. Clinically relevant fractionated schedules of 20 × 2 Gy to the carotid artery resulted in a similar carotid plaque phenotype in *ApoE*$^{-/-}$ mice compared to a single-dose treatment (14 Gy) [9]. Furthermore,

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http://dx.doi.org/10.1016/j.radonc.2014.01.006
0167-8140/

local cardiac irradiation of $ApoE^{-/-}$ mice induced an inflammatory response and microvascular damage, and enhanced atherosclerosis development in coronary arteries [11].

In the absence of monocytes/macrophages, hypercholesterolemia is not sufficient to drive the pathologic process of atherosclerosis, indicating that macrophages exert essential functions during atherogenesis [12]. They are the major cellular components of an atherosclerotic lesion and can impact plaque progression and stability by producing inflammatory mediators, regulating cholesterol metabolism, recruiting vascular smooth muscle cells (vSMCs), inducing lipid necrotic core formation, and producing matrix metalloproteinases (MMPs) and reactive oxygen species (ROS) [13,14]. Macrophages have remarkable plasticity that allows them to efficiently respond to different micro-environmental signals and to change their phenotype. Their physiology can also be markedly altered by both innate and adaptive immune responses [15,16]. In the last few years, it has become accepted that macrophages can reversibly polarize into two main phenotypes, classically- (M1 phenotype) and alternatively-activated (M2 phenotype) macrophages, responsible for promoting and resolving inflammation respectively [17–20]. Classically-activated macrophages are generated by pro-atherogenic stimuli and support a T-helper $(Th)_1$-immune response, by producing pro-inflammatory cytokines as well as ROS and nitrogen intermediates through inducible nitric oxide synthase (iNOS) expression. In contrast, alternatively-activated macrophages are induced by $Th_2$-related cytokines and secrete anti-inflammatory cytokines and upregulate scavenger receptors and arginase-1. M1- and M2-macrophage subsets have been identified in multiple pathological settings [21,22], including experimental and human atherosclerotic lesions [23–25]. Due to the very heterogeneous plaque micro-environment, new plaque-specific macrophage phenotypes, such as Mox and M4 macrophages, were recently discovered [26–28].

In developed countries, almost all individuals have subclinical atherosclerotic lesions even at young age, and although cancer may affect people of all ages, the risk for the more common varieties of cancer increases with age. Moreover, Mitchel et al. have recently stressed the importance of atherosclerotic disease stage in determining the effect of radiation exposure on progression of atherosclerosis [29]. Therefore, we here investigated the effect of local radiotherapy on pre-existing atherosclerotic lesions in the aortic arch of aged $ApoE^{-/-}$ mice.

## Material and methods

### *Mice and irradiation procedure*

At an age of 28 weeks and an average body weight of 33.2 g ± 1.1, $ApoE^{-/-}$ mice on a C57BL/6J background were randomly allocated to receive irradiation or sham-treatment. The mice were housed in filter-top cages and provided ad libitum with standardized mouse chow containing 3.7% fat (RM1 (E) SQC, SDS London, UK) and with drinking water. A total of 46 mice were included in the quantitative analysis of atherosclerotic lesions, 22 mice were sacrificed 4 weeks after treatment (10 mice were used as control, 12 mice received irradiation) and 24 mice were sacrificed 12 weeks after treatment (unirradiated control and irradiated groups contained each 12 mice).

Mice were locally irradiated with a single dose of 14 Gy[1] (250-kV X-rays at 12 mA, filtered with 0.6 mm copper) to the neck region encompassing carotid arteries, aortic arch and base of the heart, as described before [10], or received sham-treatment (0 Gy). During irradiation or sham-treatment, mice were immobilized in perspex jigs with the non-target areas shielded with lead.

Experiments were in agreement with the national regulations for animal experiments and the local animal welfare committee approved all experimental protocols.

### *Tissue preparation*

Immediately after sacrifice the arterial system was perfused with 0.1 mg/ml sodium-nitroprusside in phosphate-buffered saline (PBS), followed by 1% paraformaldehyde. The cervical, thoracic and abdominal arterial tree was excised and fixed for 24 h in 1% paraformaldehyde before transfer to 70% ethanol. The aortic arch was embedded in paraffin and 4 μm longitudinal, serial sections were cut and numbered sequentially.

### *Morphometric analysis of plaque*

Every fifth section of the aortic arch was stained with hematoxylin and eosin (H&E) and examined for the presence of atherosclerotic plaques, as described previously [10]. These plaques were categorized as initial lesions (macrophage-rich without a thick fibrous cap) or advanced lesions (well-defined necrotic core or thick fibrous cap) based on the criteria by Virmani et al. [30]. The mean number of initial and advanced lesions in the brachiocephalic artery was determined 4 and 12 weeks after 0 or 14 Gy.

Morphometric parameters were analyzed using a microscope coupled to a computerized morphometry system (Leica Qwin V3, Leica, The Netherlands). All measurements were done by one investigator (KG), without prior knowledge of the treatment group. The intra-observer variation was less than 10%. Plaque and necrotic areas (expressed as percentage of individual plaque area) were measured on four selected sections that cover the central part of the brachiocephalic artery lesion (present in 90% of the mice) and the average of these measurements was determined.

The collagen content, based on a Sirius Red staining, was analyzed on two selected sections that cover the central part of the advanced brachiocephalic artery lesion. The relative collagen content was calculated by dividing the area of collagen by the individual plaque area. The average collagen content was determined per lesion.

Evaluation of thrombotic characteristics of plaques in the aortic arch was performed on one of the central sections and scored semi-quantitatively. Sections were examined for the presence of fibrin deposits (Martius-Scarlet-Blue Trichrome staining) and erythrocyte- (H&E staining) or iron-containing macrophages (Perl's staining), as an indication of previous intraplaque hemorrhage.

### *Immunohistochemistry*

One central section per brachiocephalic artery lesion was stained with MAC3-antibody (1:30, Becton & Dickinson, USA), cleaved caspase-3-antibody (1:100, Cell Signaling, USA) and CD45-antibody (1:5000, Becton & Dickinson, USA) to detect macrophages, apoptotic cells and leukocytes, respectively. Results were expressed as number of antibody-positive cells relative to the average plaque area and group means were calculated. Furthermore, a staining with rabbit-anti-mouse iNOS (1:20, Abcam, UK) and rabbit-anti-mouse arginase-1 (1:500, kindly provided by Paul van Dijk, department of Anatomy and Embryology, Maastricht University, The Netherlands) was performed to semi-quantitatively score the presence of respectively M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophages in the plaque (grading no presence, presence or high presence).

[1] Assuming an $\alpha/\beta$ ratio of 2–3 Gy for late vascular damage, a single dose of 14 Gy is approximately equivalent to 48–56 Gy in 2 Gy fractions, according to the Linear Quadratic formula:

$$EQD2 = D(d + (\alpha/\beta)/2 + (\alpha/\beta))$$

EQD2 = equivalent dose in 2 Gy fractions; D = total dose; d = dose per fraction.