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Effect of irradiation and bone marrow transplantation on angiotensin II-induced aortic inflammation in ApoE knockout mice

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ABSTRACT

Background and aims: Angiotensin II (Ang II) infusion promotes the development of aortic aneurysms and accelerates atherosclerosis in *ApoE*$^{-/-}$ mice. In order to elucidate the role of hematopoietic cells in these pathologies, irradiation and bone marrow transplantation (BMT) are commonly utilized. The aim of this study was to investigate the effects of irradiation and BMT on abdominal and thoracic aortic aneurysm formation and acute leukocyte recruitment in the aortic root and descending aorta, in an experimental mouse model of aortic aneurysm formation.

Methods: *ApoE*$^{-/-}$ mice were either lethally irradiated and reconstituted with *ApoE*$^{-/-}$ bone marrow or non-irradiated. Following engraftment, mice were treated with Ang II to induce aortic inflammation and accelerate atherosclerosis.

Results: Ang II infusion (0.8 mg/kg/day) in BMT mice resulted in reduced aortic aneurysms and atherosclerosis with decreased leukocyte infiltration in the aorta compared to non-BMT mice, when receiving the same dose of Ang II. Furthermore, the reduced aortic infiltration in BMT mice was accompanied by increased levels of monocytes in the spleen and bone marrow. A dose of 3 mg/kg/day Ang II was required to achieve a similar incidence of aneurysm formation as achieved with 0.8 mg/kg/day in non-BMT mice.

Conclusions: This study provides evidence that BMT can alter inflammatory cell recruitment in experimental mouse models of aortic aneurysm formation and atherosclerosis and suggests that irradiation and BMT have a considerably more complex effect on vascular inflammation, which should be evaluated.

1. Introduction

Angiotensin II (Ang II) infusion into *ApoE*$^{-/-}$ mice augments atherosclerotic plaque and abdominal aortic aneurysm (AAA) formation [1], via mechanisms causing increased monocyte-macrophage recruitment and vascular wall remodeling. Accordingly, Ang II-induced vascular inflammation can be studied by treating hyperlipidaemic mice with Ang II to investigate long-term, chronic inflammatory responses such as plaque formation, or short-term acute inflammatory processes such as cellular infiltration. To explore the role of hematopoietic cells in disease models, irradiation and bone marrow transplantation (BMT) have been used as a tool to generate chimeric mice, receiving donor bone marrow cells from transgenic mice harboring a genetic alteration in a functional pathway [2].

For BMT experiments, bone marrow cells are isolated from the donor mouse and injected (usually intravenously) into the recipient mouse, which has been irradiated to ablate its bone marrow cells.

Following successful BMT, the hematopoietic system is generally repopulated within 4 weeks [2,3]. The effect of BMT on the susceptibility to atherosclerosis has indicated that irradiation has more complex outcomes on plaque formation. In BMT studies in LDL-receptor deficient mice fed a high fat diet, aortic root lesions were greater in the BMT mice, while the lesions in the descending aorta were greater in the non-BMT mice [4]. In line with the a

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former observation, other BMT studies in *ApoE*$^{-/-}$ mice have suggested that high levels of irradiation or fractionated irradiation accelerates the development of macrophage-rich, inflammatory atherosclerotic lesions in the carotid arteries [5,6]. However, the direct effects of irradiation and BMT on Ang II-induced aortic aneurysm formation and Ang II-induced atherosclerosis has not be examined.

Bone marrow transplantation (BMT) following irradiation is commonly used in experimental studies designed to investigate the specific contribution of BM-derived circulating cells to inflammatory disease processes. During previous studies investigating the formation of abdominal aortic aneurysms (AAA) induced by Ang II in *ApoE*$^{-/-}$ mice that had undergone irradiation and BMT to generate chimeric mice with different gene expression in BM-derived cells *versus* host cells, we observed that irradiation and BMT itself affects the degree of AAA development and rupture in *ApoE*$^{-/-}$ mice receiving *ApoE*$^{-/-}$ bone marrow. In this current study, we further investigated the effects of BMT on aortic inflammatory disease and accelerated atherosclerosis in male *ApoE*$^{-/-}$ mice treated with Ang II. Here, we report that aneurysm formation in BMT *ApoE*$^{-/-}$ mice treated with Ang II was absent and required an increase in the dose of Ang II to achieve the same degree of aneurysm formation and rupture to that of non-BMT *ApoE*$^{-/-}$ mice treated with Ang II, highlighting the need to interpret BMT and Ang II-induced AAA studies more critically.

2. Materials and methods

2.1. Mice

ApoE$^{-/-}$ mice were housed in individually ventilated cages with 12 h light/dark cycle and controlled temperature (20 °C - 22 °C). Standard chow (B & K Universal Ltd, UK) and water were available *ad libitum*. All animal studies were conducted with ethical approval from the Local Ethical Review Committee and in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986.

2.2. Bone marrow transplantation (BMT)

Mice were irradiated (2×5 Gy) and transplanted with bone marrow cells (5×10^6) at the age of 10 weeks. As a control for the efficiency of the irradiation procedure and bone marrow transfer we performed rigorous testing to characterise and confirm chimerism. We used mouse strains with alternate CD45.1 or CD45.2 leukocyte antigens to allow 'tracking' of engrafted cells from the donor in the chimeric recipient after marrow ablation by irradiation. We confirmed that more than 95% blood cells in recipient mice are donor-derived 6 weeks after transplantation, with reconstitution of normal peripheral leukocyte counts and subset profiles confirming successful reconstitution (Supplementary Figure 1). Hereafter, BMT will indicate bone marrow transplantation, which is an all-inclusive term for the accompanying irradiation as well as the bone marrow reconstitution.

2.3. Ang II infusion, blood pressure recordings and aneurysm characterisation

For these studies, we used the Ang II infused *ApoE*$^{-/-}$ C57/Bl6 mouse model developed in the laboratory of Daugherty et al. [1]. In this mouse model, suprarenal AAAs form in up to 80–85% of the cases. As indicated in Robinet et al., there is a low AAA incidence in Ang II infused female *ApoE*$^{-/-}$ mice (~20%), therefore we chose to study male *ApoE*$^{-/-}$ mice that have between 80 and 100% AAA incidence [7]. Both sex hormones [8] and sex chromosomes [9] have been shown to effect AAA development in experimental AAA. Once, successful reconstitution was confirmed after 6 weeks of recovery, mice were infused with Ang II for 5 or 14 days. Systolic blood pressure was measured on day 2 – day 14 of Ang II or saline infusion. Briefly, BMT or non BMT male mice were anaesthetized with isoflurane by inhalation and osmotic mini-pumps (Alza Corp, USA) delivering Ang II (0.8, 1.5, or 3 mg/kg/day; Sigma-Aldrich, UK) were implanted subcutaneously. Systolic blood pressure was measured using an indirect method of blood pressure measurement in animals, using a non-invasive computerized tail-cuff system in conscious mice following a 1 week training period (Visitech BP2000, Visitech Systems, Inc., USA) following guidelines in Kurtz et al. [10]. We categorised aortic aneurysms as remodelled tissue in the supra-renal and/or thoracic regions of the aorta that contained thrombus of surviving mice at harvest (Type II-Type IV). Aortic aneurysm rupture was characterised by blood in the cavity and large thrombi in the aorta of mice that were found dead. 'No aneurysm' was characterised by the absence of any thrombi and the absence of large dilatation in the supra-renal and/or thoracic regions of the aorta of surviving mice at harvest. Maximal outer widths of suprarenal abdominal aortas were measured using Image Pro Plus software (Media Cybernetics, USA) by a researcher blind to the group assignment.

2.4. Atherosclerosis analysis

All animal atherosclerosis measurements were based on the recommendations in Daugherty et al. [11]. *ApoE*$^{-/-}$ mice were fed a normal mouse chow diet (19.67% crude protein, 4.13% crude oil and 3.22% crude fibre; B & K Universal Ltd, UK) and atherosclerotic lesion size was assessed in paraffin-embedded aortic root sections stained with Masson-Goldner trichrome (Merck, Germany). Serial sections of 7 μm thickness were cut with a microtome (Leica RM2155; Leica Microsystems) from the first appearance of the tricuspid valves until valves were no longer visible and mounted onto adhesive poly-L-lysine coated slides (VWR, Leicestershire, UK). Approximately 60–80 sections were cut for each animal and anatomical markers were used to enable sections from 3 depths within the cusp region to be taken at the same depth in each animal for staining. The average lesion size was calculated from three sections taken at 100 μm intervals starting from the section showing all three aortic cusps. For immunohistochemical staining, serial sections of aortic roots were assessed for macrophage content using an anti-Galectin-3 antibody (BD Pharmingen, UK). As controls for positive staining, paraffin embedded liver sections were stained in parallel experiments for Galectin-3. For negative controls, one section on each slide for each animal was incubated with the respective isotype antibody to determine staining specificity. Aortic roots were visualised and imaged (coolSNAP-pro camera, Roper Scientific, Leica DMRBE microscope and the lesion area and Galectin-3 positive areas quantified from digitized microscopic images using Image-Pro Plus (Media Cybernetics, USA).

2.5. Flow cytometry

Descending aortas from the aortic arch to femoral bifurcations that did not contain a visible aneurysm were micro-dissected and digested in an enzyme solution containing 60 U/ml DNase I, 60 U/ml Hyalronidase, 450 U/ml Collagenase I and 125 U/ml Collagenase XI (all enzymes from Sigma-Aldrich, UK) at 37 °C as described in Galkina et al. [12]. A single cell suspension was prepared by passing aortic pieces through a strainer for subsequent flow cytometry staining. Isolated aortic cells were stained with monoclonal antibodies directed against CD45+, CD11b+, Ly6C+, Ly6G–(monocytes) and CD45+, CD11b+, Ly6C+, Ly6G+ (neutrophils) (BD Pharmingen) with appropriate isotype controls as described in Tieu et al. [13].