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Vaccination against TIE2 reduces atherosclerosis

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ABSTRACT

Background: TIE2$^+$ cells play a crucial role in processes that are involved in atherosclerosis, such as angiogenesis. Therefore, the specific deletion of TIE2$^+$ cells by means of DNA vaccination may affect atherosclerosis.
Methods: Cellular immunity against cells that overexpress TIE2 was established in LDLr$^{-/-}$ mice by a novel oral DNA vaccination technique, in which an attenuated *Salmonella typhimurium* strain was used as a carrier for plasmid pcDNA3.1 encoding TIE2. After three oral vaccinations with 2-week time intervals LDLr$^{-/-}$ mice were put on a Western type diet and atherosclerosis was induced.
Results: Eight weeks after vaccination FACS analysis of circulating peripheral blood mononuclear cells (PBMCs) revealed a significant decrease (33%, $p < 0.05$) in TIE2$^+$ cells upon vaccination against TIE2, indicating the successful induction of cellular immunity following vaccination against TIE2. Six weeks after collar placement vaccination against TIE2 resulted in significantly decreased carotid atherosclerosis, as indicated by 30% ($p < 0.05$) reduced intima area and 27% ($p < 0.05$) reduced intima/lumen ratios. Furthermore, atherosclerosis was attenuated in the aortic root by 42% ($p < 0.05$), further underlining the anti-atherosclerotic effect of vaccination against TIE2. Adventitial angiogenesis was reduced by 61% ($p < 0.05$) upon vaccination against TIE2 providing a mechanism via which vaccination against TIE2 inhibits lesion formation. Histochemical analysis of the atherosclerotic lesion composition revealed a 1.6-fold (carotid artery, $p < 0.05$) and 1.9-fold (aortic root, $p < 0.05$) increase in collagen content upon vaccination against TIE2, indicating a more stable plaque phenotype.
Conclusions: We demonstrate that vaccination against TIE2 induces cellular immunity against cells that overexpress TIE2 and results in smaller atherosclerotic lesions with a more stable phenotype. Therefore, vaccination strategies that target cells that contribute to atherosclerosis, may be of potential use in the development of novel treatments of atherosclerosis.

1. Introduction

Despite the use of lipid lowering therapies, atherosclerosis remains the major cause of cardiovascular disease, which indicates the need to develop novel strategies to treat atherosclerosis. Accumulating data suggest that vaccination may be an attractive method to induce long-lasting protection against endogenous factors that contribute to the development of atherosclerosis. Along this line, vaccination against modified low-density lipoprotein (LDL) [1], apolipoprotein B100 (apoB100) [2], cholesteryl ester transfer protein (CETP) [3], and interleukin 12 (IL-12) [4], has been proven to successfully reduce atherosclerosis in mouse studies. Furthermore, it was suggested that a cell-based approach, in which the amount of anti-atherosclerotic cells is increased or in which the amount or the recruitment of pro-atherosclerotic cells is reduced, may contribute to the development of a novel therapy against atherosclerosis [5,6]. Recently, we combined these two strategies to treat atherosclerosis by vaccination against cells that overexpress vascular endothelial growth factor receptor 2 (VEGFR2), which resulted in CD8$^+$ T cell driven specific lysis of VEGFR2$^+$ cells [7,8]. These VEGFR2$^+$ cells contribute to processes that are involved in atherosclerosis, such as angiogenesis [9–11] and vaccination against VEGFR2 resulted in a significant reduction in initiation and progression of atherosclerosis [7].

In the present study, we aimed to substantiate our hypothesis that targeting of pro-atherosclerotic cells by vaccination may be of potential use in the treatment of atherosclerosis. To that end we focused in this study on TIE2 overexpressing cells. TIE2

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was recently identified as a receptor tyrosine kinase (RTK) that is expressed predominantly on endothelial (precursor) cells in both the embryonic and the adult vasculature in a wide range of tissues [12–14]. Natural ligands for TIE2 are angiopoietins, of which Ang1 induces phosphorylation of TIE2 [15,16], whereas Ang2, although context dependent, inhibits TIE2 activation [17]. The interaction of angiopoietins with TIE2 has been shown to regulate endothelial cell survival and migration, which may at least partly explain the essential role of TIE2 in vessel maturation during angiogenesis [15]. Furthermore, TIE2 has been shown to identify a hematopoietic lineage which is able to contribute to neointimal angiogenesis [18]. In addition, both Ang1 and Ang2 have been shown to increase the recruitment of leukocytes leading to enhanced proinflammatory responses [19]. TIE2 expression is not restricted to endothelial cells but some of the subendothelial intimal cells have been shown to be double positive for TIE2 and smooth muscle cell markers, but negative for endothelial markers [20,21]. Moreover, Ang1 has been shown to promote adhesion of monocytes to vascular smooth muscle cells and increase plaque size in apoE$^{-/-}$ mice [22]. Consequently, TIE2^{+} cells form an interesting target for interference in atherosclerosis and a reduction in their number may be beneficial for the outcome of atherosclerosis.

Therefore, we induced T-cell-mediated immunity against TIE2 overexpressing cells by the previously described oral DNA vaccination technique [7,8], in which an attenuated strain of *Salmonella typhimurium*, carrying a plasmid encoding TIE2, is used as the vaccine. By inducing cellular immunity against TIE2 by vaccination we aimed to inhibit atherosclerosis and to induce a more stable plaque phenotype. In this way we aimed to designate this vaccination strategy as a potential novel way to treat atherosclerosis.

2. Methods

2.1. Vaccination strategy

Plasmid pcDNA3.1 (Invitrogen, CA, USA) encoding murine TIE2 was constructed and electroporated in attenuated *S. typhimurium* Aro/A (strain SL7207) bacteria, as previously described [8]. Mice were vaccinated by three times oral administration (with 2-week intervals) of 1×10^8 *S. typhimurium* bacteria transformed with either pcDNA3.1-TIE2 or pcDNA3.1 (control) in 100 μl PBS, as described previously [8].

2.2. Induction of atherosclerosis

All animal work was performed in compliance with the guidelines issued by the Dutch government. Female LDLr$^{-/-}$ mice, aged 10–12 weeks, were vaccinated against TIE2 ($n = 11$) or control vaccinated ($n = 12$) by three times oral administration of the vaccine with 2-week intervals. All experiments were performed with these two groups of mice. Immediately after the last administration of the vaccine, mice were put on a Western type diet, containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, UK). After 2 weeks of Western type diet feeding, mice were anesthetized and atherosclerosis was induced in carotid arteries by bilateral perivascular collar placement, as described previously [23]. Six weeks after collar placement the mice were sacrificed for analysis of the atherosclerotic lesions. Furthermore, immediately after sacrifice the induction of cellular immunity against TIE2 overexpressing cells was confirmed by quantification of the amount of circulating TIE2^{+} cells.

2.3. Quantification of circulating TIE2^{+} cells by flow cytometry

At the moment of sacrifice (8 weeks after the last vaccination) blood was collected from the orbital sinus after induction of general anesthesia. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood on a density gradient (Lympholyte, Cedarlane Laboratories, Canada) according to manufacturer's instructions. PBMCs (2×10^5/well) were stained with a phycoerythrin (PE) conjugated antibody against TIE2 (clone TEK4, Immunosource, The Netherlands), and with a FITC-conjugated antibody against CD34 (Clone RAM34, Immunosource) for 45 min with a concentration of 2 μg/ml. After three washing steps with PBS 5×10^4 cells were analyzed with a Becton Dickinson FACS Calibur flow cytometer.

2.4. Tissue harvesting

Carotid arteries and hearts were isolated after *in situ* perfusion for 15 min with PBS and sequent perfusion with formalin for 30 min. Fixated carotids were embedded in OCT compound (Sakura Finetek, The Netherlands), snap-frozen in liquid nitrogen, and stored at −20 °C until further use. Transverse 5 μm cryosections were prepared in a proximal direction from the carotid bifurcation and mounted on a parallel series of slides. For analysis of atherosclerosis at the site of the tricuspid valves in the aorta 10 μm transverse cryosections were made of the aortic root.

2.5. Histochemistry

Cryosections were routinely stained with hematoxylin (Sigma Diagnostics, MO, USA) and eosin (Merck Diagnostica, Germany). Corresponding sections were stained immunohistochemically with antibodies against a macrophage specific antigen (MOMA-2, polyclonal rat IgG_{2b}; Research Diagnostics Inc., NJ, USA) or α-smooth muscle cell actin (monoclonal mouse IgG_{2a}, clone 1A4; Sigma Diagnostics). Sections were incubated with primary antibodies for 2 h. As secondary antibodies, goat anti-mouse IgG peroxidase conjugate (Nordic, The Netherlands), or goat anti-rat IgG alkaline phosphatase conjugate (Sigma Diagnostics) were used, with 3,3′-diamino-benzidine (Sigma Diagnostics) or 5-bromo-4-chloro-3-indolyl phosphate (Sigma Diagnostics) as enzyme substrates. Sections were stained for CD31 with a rat anti-mouse antibody (clone MEC13.3, dilution 1:50, Pharmingen, CA, USA) and for CD3 (molecular complex 17A2, BD Pharmingen) combined with a biotynilated polyclonal anti-rat secondary antibody (dilution 1:100, Pharmingen). For visualization the signal was amplified by the CSA system (Dako, The Netherlands). Corresponding sections were stained for collagen by picrosirius red (Direct red 80) staining. Cryosections of lesions in the aortic root were stained for lipids with an Oil red O staining.

2.6. Morphometry

Hematoxylin–eosin-stained sections of carotid arteries were used for morphometric analysis of atherosclerotic lesions. Sections were analyzed using a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, UK). Each carotid artery was assessed ∼0.5 mm proximal to the collar, and the site of maximal stenosis was used for morphometric analysis [23]. Contents of macrophages, smooth muscle cells, T lymphocytes and collagen were obtained by dividing the area of specific staining within the intima by the total intima area. Analysis of atherosclerotic lesion area in the aortic root was performed on the Oil red O stained sections, as previously described [24]. Quantification of adventitial neovessel formation was performed by counting the number of CD31 stained capillar-