Vaccination Against VEGFR2 Attenuates Initiation and Progression of Atherosclerosis

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Objective—Vascular endothelial growth factor receptor 2 (VEGFR2)–overexpressing cells may form an interesting target for the treatment of atherosclerosis because of their involvement in processes that contribute to this disease, such as angiogenesis.

Methods and Results—We vaccinated mice against VEGFR2 by an orally administered DNA vaccine, comprising a plasmid, encoding murine VEGFR2, carried by live attenuated Salmonella typhimurium. This vaccine induces cellular immunity against cells that overexpress VEGFR2. Vaccination of hypercholesterolemic mice against VEGFR2 resulted in a marked induction of CD8+ cytotoxic T cells specific for VEGFR2 and led to an inhibition of angiogenesis in a hindlimb ischemia model. Interestingly, VEGFR2 vaccination attenuated the progression of preexisting advanced atherosclerotic lesions in the brachiocephalic artery of apoE−/− mice. Furthermore, VEGFR2 vaccination strongly reduced the initiation of collar-induced atherosclerosis in the carotid arteries of LDLr−/− mice. In addition, denudation of the carotid artery, as a model for postinterventional lesion formation, resulted in delayed endothelial replacement and significantly increased neointima formation on VEGFR2 vaccination.

Conclusions—These data indicate the prominent role of VEGFR2+ cells in cardiovascular diseases and show that induction of cellular immunity against atherosclerosis-associated cells by means of DNA vaccination may contribute to the development of novel therapies against atherosclerosis. (Arterioscler Thromb Vasc Biol. 2007;27:2050-2057.)

Key Words: atherosclerosis • restenosis • vaccination • angiogenesis • VEGFR2+ cells

Atherosclerosis is a multifactorial disease, which is regulated by a variety of inflammatory mechanisms.1 Many cell types contribute to the initiation and progression of the atherosclerotic plaque. The process of atherosclerosis is initiated by the activation of endothelial cells on exposure to various stimuli, such as high levels of atherogenic lipoproteins or shear stress, leading to enhanced migration of leukocytes from the circulation into the intima. The accumulation and activation of macrophages with subsequent foam cell formation after uptake of atherogenic lipoproteins within the intima are key processes that induce the formation of an atherosclerotic plaque. Changes in proliferation, migration, and activation of smooth muscle cells (SMCs) also regulate the process of atherosclerosis. More recently identified inflammatory regulators in atherosclerosis are T cells, B cells, dendritic cells, and NKT cells.1

In the present study, we focused on the role of a specific cell population in atherosclerosis, that highly expresses vascular endothelial growth factor receptor 2 (VEGFR2), and we aimed to diminish the amount of these cells and study the effect thereof on atherosclerosis. Highly VEGFR2 expressing cells comprise proliferating endothelial cells and (endothelial) progenitor cells,2 which are involved in angiogenesis.3–5 A process that potentially contributes to the initiation and progression of atherosclerotic plaques.6–7 Furthermore, VEGFR2 is highly expressed on the activated endothelial cell layer that covers the atherosclerotic plaque, whereas the healthy vascular wall shows much lower expression levels of VEGFR2 on the luminal endothelial cells.8 In addition, some studies indicate that VEGFR2+ progenitor cells or endothelial cells can differentiate into nonendothelial cells, such as SMCs that may contribute to atherosclerosis.9,10 Moreover, VEGF has been demonstrated to promote the progression of atherosclerosis and lead to increased macrophage content in the atherosclerotic plaque, indicating that interference in the interaction of VEGF with its main receptor, VEGFR2, may
result in reduced atherogenesis. These proatherogenic effects are substantiated by the fact that binding of VEGF to VEGFR2 induces inflammatory responses in endothelial cells by NF-κB activation, leading to elevated expression levels of vascular endothelial cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and endothelial selectin (E-selectin) in endothelial cells resulting in increased adherence of monocytes. In the present study, we targeted cells that overexpress VEGFR2 with a novel DNA vaccination technique, which has recently been used to block tumor angiogenesis leading to dramatically reduced tumor growth and prolonged survival of mice that were challenged by several tumor cell lines. By oral administration of the live attenuated bacterium, *Salmonella typhimurium*, containing a VEGFR2-encoding plasmid, we induced a cytotoxic CD8+ T cell response against VEGFR2 overexpressing cells in atherosclerosis prone mice. We show that vaccination against VEGFR2 attenuates both the initiation and progression of atherosclerosis, but leads to enhanced postinterventional neointima formation, which illustrates the important role of VEGFR2 overexpressing cells in stenotic vascular processes. Furthermore, we show for the first time that targeting of specific subpopulations of cells by vaccination may be a valuable strategy in the development of novel therapies against atherosclerosis.

**Methods**

For detailed Methods please see the supplemental materials (available online at http://atvb.ahajournals.org).

**Vaccination Strategy**

Plasmid pcDNA3.1 (Invitrogen) encoding murine VEGFR2 was constructed and electroporated in attenuated *S typhimurium* Aro/A (strain SL7207) bacteria, as previously described.

**Induction of VEGFR2-Specific Cytotoxic CD8+ T Lymphocytes**

The potential of splenocytes, isolated from vaccinated or control apoE-/- mice 3 weeks after the last administration of the vaccine, to lyse VEGFR2-expressing cells was determined in a standard 51Cr release assay.

**IFN-γ and VEGF ELISA**

Serum IFN-γ levels and serum VEGF levels were determined using the Femto-High Sensitivity IFN-γ ELISA Kit (eBioscience) and a VEGF ELISA kit from Biosource.

**Angiogenesis in a Hindlimb Ischemia Model**

For evaluation of antiangiogenic effects induced by vaccination against VEGFR2, apoE-/- mice were vaccinated and unilateral hindlimb ischemia was introduced as described.

**Induction of Atherosclerosis**

The effect of vaccination against VEGFR2 on early and late stages of atherosclerosis was studied in 2 mouse models for atherosclerosis.
Apoe\(^{-/-}\) mice and Ldlr\(^{-/-}\) were acquired from TNO-PG (Leiden, The Netherlands) and were used to study progression and initiation of atherosclerosis, respectively.\(^{15}\)

**Induction of Postinterventional Neointima Formation**

Female 3-month-old Ldlr\(^{-/-}\) mice were vaccinated (VEGFR2 vaccinated: \(n=15\), control vaccinated: \(n=18\)) as described. Two weeks after the last immunization mice were put on a Western type diet. One week later, mice were anesthetized and endothelial denudation and analysis of the right common carotid artery was performed as described.\(^{16}\)

**Tissue Harvesting, Histology and Immunohistochemistry**

Cryosections of arteries were routinely stained with hematoxylin and eosin. Staining for actin, MOMA2, CD31, VEGFR2, collagen, and IFN-\(\gamma\) was performed as described. Quantification of atherosclerosis and restenosis was performed as described.

**Data Analyses**

Values are expressed as mean±SEM. A 2-tailed Student \(t\) test was used to compare individual groups of mice or cells. A level of \(P<0.05\) was considered significant.

**Results**

**Vaccination Induces VEGFR2 Specific CD8\(^{+}\) Cytotoxic T Cells**

Three weeks after the third vaccination of apoe\(^{-/-}\) mice by oral administration of \(S\) typhimurium transformed with pcDNA3.1-VEGFR2 or pcDNA3.1, splenocytes and CD8\(^{+}\) T cells were isolated to determine their capacity to specifically kill VEGFR2-expressing cells in a standard \(^{31}\)Cr release assay. We observed a significant 2.4- to 3.9-fold increase in effector/target ratio dependent lysis of VEGFR2\(^{+}\) 3T3 cells by splenocytes from VEGFR2 vaccinated mice (Figure 1A, \(P<0.001\)) as compared with splenocytes from control vaccinated mice. Incubation with CD8\(^{+}\) T cells, isolated from VEGFR2 vaccinated mice, in a 50:1 effector/target cell ratio resulted in a 3.68-fold increased cytotoxicity (Figure 1B, \(P<0.001\)) as compared with incubation with CD8\(^{+}\) T cells from control mice, indicating that the cytotoxic effect of the spleen cells can be attributed to the CD8\(^{+}\) cells. A significant decrease in the number of VEGFR2\(^{+}\)/GFP\(^{+}\) cells was seen on exposure to splenocytes from VEGFR2 vaccinated mice, whereas splenocytes from control mice did not affect the survival (Figure 1C through 1E). Vaccination against VEGFR2 did not affect anti-VEGFR2 IgG antibodies in serum (data not shown).

**Antiangiogenic Effect of Vaccination Against VEGFR2**

Next we determined the effect of lysis of VEGFR2 overexpressing cells by VEGFR2 vaccination on angiogenesis. The left femoral artery of apoe\(^{-/-}\) mice was coagulated unilaterally 1 week after the third vaccination to induce ischemia. Two weeks later ischemia-induced angiogenesis was quantified by measuring the CD31-positive area. Vaccination against VEGFR2 resulted in a 2.0-fold decrease in CD31-positive area as compared with control vaccination (Figure 2A through 2C; 3422±581 versus 1743±168 \(\mu\)m\(^2\), \(P<0.01\)), whereas serum levels of VEGF were not affected after vaccination (Figure 2D; 51.2±6.4 versus 49.2±6.0 ng/mL), indicating that the antiangiogenic effect is VEGF-independent.

**Effect of Vaccination Against VEGFR2 on Atherosclerosis**

The effect of vaccination against VEGFR2 on advanced lesions was determined using apoe\(^{-/-}\) mice (22 weeks of age), which had developed atherosclerotic lesions on chow diet. A group of control-vaccinated mice was euthanized directly after the last vaccination and lesion formation was quantified in the brachiocephalic artery and compared with lesion formation 12 weeks after vaccination against VEGFR2 or control vaccination. Vaccination against VEGFR2 significantly reduced the plaque size 12 weeks after vaccination by 25% compared with control vaccinated mice (Figure 3A through 3C; 283 100±15 120 versus 211 767±21 433 \(\mu\)m\(^2\), \(P<0.05\)), whereas cholesterol and triglyceride levels remained unchanged in all groups. However, when the initial level of atherosclerosis at the time of the last vaccination is taken into account (Figure 3, gray line [176 098 \(\mu\)m\(^2\)]), it is clear that the progression of atherosclerosis was dramatically reduced by 66% on VEGFR2 vaccination.

Collar-induced atherosclerosis in Ldlr\(^{-/-}\) mice fed a Western type diet was used to determine the effect of vaccination on the initiation of atherosclerosis. Vaccination against VEGFR2 reduced the initiation of atherosclerosis by 77% (Figure 4D, \(P<0.05\)), which translated into a marked 2.7-fold reduction in intima/ media ratio (Figure 4E, \(P<0.01\)) and a 3.0-fold decreased intima/lumen ratio (Figure 4F, \(P<0.01\)). In addition VEGFR2 vaccination significantly diminished media area, whereas we did not observe significant differences in macrophage, SMC, collagen, or IFN-\(\gamma\) content (Figure 5G through 5H) or serum IFN-\(\gamma\)-levels (supplemental Results and supplemental Figure I).

**VEGFR2\(^{+}\) Cells and Neovessels in Atherosclerosis**

The presence of neovessels in atherosclerosis-affected arteries was assessed by CD31 staining. Apart from
endothelial cells covering the atherosclerotic plaque (Figure 5A), CD31
/H11001 cells were not found within the neointima, but CD31
 vessels were found in the media and adventitia underlying the atherosclerotic lesion (Figure 5B) and were more abundant as compared with arteries in which atherosclerosis was not induced. Many of the latter microvessels were positive for VEGFR2 (Figure 5C and 5D), whereas VEGFR2
 cells were frequently identified in the adventitia without association with microvessels (Figure 5E). Apart from occasional VEGFR2
 endothelial cells covering the atherosclerotic lesion, no VEGFR2
 cells were found within the atherosclerotic plaques of carotid or brachiocephalic arteries (Figure 5F).

Effect of VEGFR2 Vaccination on Postinterventional Neointima Formation
The endothelial layer of carotid arteries was denudated in LDLr
 mice and vascular wall or neointima formation was

Figure 4. Vaccination against VEGFR2 inhibits initiation of atherosclerosis. While plasma cholesterol levels remained unaffected (A), 6 weeks after collar placement intima area was reduced by 77% on vaccination against VEGFR2 (black bars, C and D) as compared with control-vaccinated mice (open bars, B), which translated to 2.7- and 3.0-fold decreases in intima/media (E) and intima/lumen ratios (F), respectively. *P<0.05, **P<0.01.

Figure 3. Effect of vaccination against VEGFR2 on preexisting atherosclerotic lesions. Brachiocephalic lesion size in apoE
 mice 1 day after the last vaccine administration is indicated by the gray line (A). Twelve weeks later, vaccination against VEGFR2 (black bars) resulted in a 25% reduction in lesion size as compared with control-vaccinated mice (open bars; A), or 66% reduction in progression of atherosclerosis since the last immunization (B and C). *P<0.05.
analyzed 1 and 5 weeks later to evaluate the effect of vaccination against VEGFR2 on postinterventional neointima formation. One week after denudation the degree of endothelial replacement was quantified by counting the luminal flat-shaped hematoxylin stained nuclei of CD31-positive–stained cells at the site of maximal media area (supplemental Figure II) and a 31% reduction in endothelial cells after vaccination against VEGFR2 was observed (43.8 ± 4.3 versus 30.2 ± 3.3, P < 0.05; supplemental Figure IIC). Furthermore, vaccination against VEGFR2 resulted in a 1.4-fold increase in media area 1 week after denudation (34 026 ± 1020 versus 47 657 ± 4690 μm², P < 0.05; supplemental Figure IID). Five weeks after denudation neointimal lesions were stained for SMCs and showed a typical restenotic phenotype in which SMCs form the main cellular component and a significant 2.7-fold increase in lesion size on vaccination against VEGFR2 (16 028 ± 3968 versus 43 591 ± 8943 μm², P < 0.01; supplemental Figure III).

**Discussion**

Despite the use of lipid lowering, antiinflammatory, or plaque stabilizing treatments, atherosclerosis remains the major cause of cardiovascular disease, which encourages the development of alternative strategies to reduce atherosclerosis. Blockade of the recruitment, the removal of cells that contribute to atherosclerosis or the administration of antiatherosclerotic cell types, may contribute to a reduction in lesion formation.17–19

In the present study, we aimed to reduce atherosclerosis by targeting those cells that overexpress VEGFR2 by a novel DNA vaccination technique. Cell-mediated immunity against VEGFR2-overexpressing cells was induced in atherosclerosis prone mice by oral administration of *S typhimurium* carrying a plasmid encoding mouse VEGFR2.13

We confirmed that splenocytes and specifically CD8+ T cells, from VEGFR2 vaccinated mice specifically lyse cells overexpressing VEGFR2, whereas control splenocytes and control CD8+ T cells were unable to do so. A humoral response against VEGFR2 was not observed on vaccination, indicating that cellular immunity via the induction of VEGFR2-specific CD8+ T cell cytotoxicity is responsible for the observed effects. In addition, a potential VEGF-dependent mechanism is unlikely to play a role in the antiangiogenic effects of VEGFR2 vaccination because serum levels of VEGF did not change on VEGFR2 vaccination. The fact that vaccination against VEGFR2 overexpressing cells, that normally contribute to angiogenesis,2–5 inhibited hindlimb ischemia induced angiogenesis by 50% shows that the functional role of VEGFR2-overexpressing cells is impaired in vivo on VEGFR2 vaccination. These results are in line with the study of Niethammer et al in which VEGFR2 vaccination was used to block tumor growth by inhibiting tumor-related angiogenesis. In his study CD8+ T cells colocalised with the target site, the tumor vasculature.13

We now show that VEGFR2 vaccination inhibited the progression of preexisting atherosclerotic lesions in apoE<sup>−/−</sup> mice for 66%. In a second experiment we determined that vaccination against VEGFR2 reduced the initiation of atherosclerosis in LDLr<sup>−/−</sup> for 77%, which indicated that VEGFR2 vaccination already interfered with a very initial stage of atherosclerosis. We conclude that cells that highly express VEGFR2 are involved in the development of atherosclerosis and conclude that these cells regulate atherosclerosis already in a very early stage. Because several cell types overexpress VEGFR2, the effect of vaccination on atherosclerosis may be exerted by a several mechanisms. Proliferating endothelial cells show a high expression of VEGFR2 and contribute to angiogenesis,3–5 which explains the inhibiting effect on angiogenesis in the hindlimb ischemia by VEGFR2 vaccination. Angiogenesis may contribute to the progression of atherosclerosis6,7 and blockade of angiogenesis by VEGFR2 vaccination may result in decreased atherosclerosis. Microvessels, indicated by positive CD31 and VEGFR2 staining, were found in adventitia and media, but we did not identify VEGFR2 or CD31<sup>+</sup> cells within the neointima, which suggests the lack of intraplaque angiogenesis in the used models.
for atherosclerosis. This may be explained by the fact that adaptive hypoxia driven neovessel formation only occurs when (tumor) tissue is located >100 μm from the blood capillaries.20 Because in our mouse models for atherosclerosis, neointimal tissue in the brachiocephalic arteries is always within 100 μm of blood capillaries, this may explain the absence of intraplaque angiogenesis. Moulton et al21 show a 9-fold increase in the frequency of neovessels in atherosclerotic lesions, but these lesions were thicker than 250 μm.7 We did, however, observe VEGFR2 positive microvessels within the media at the site of the lesion, which may indicate the initiation of neovascularization of the lesions. These VEGFR2-positive vessels may have served as a target for the CD8+ VEGFR2-specific T cells, and this may diminish the optimal growth of the plaque on VEGFR2 vaccination. This is in line with the suggestion that adventitial angiogenesis contributes to atherosclerotic plaque growth because the degree of adventitial angiogenesis correlates with the extent of neointima formation.21,22 In addition, stimulation of adventitial angiogenesis, by administration of endothelial progenitor cells or adventitial application of VEGF, results in increased neointima formation.23,24

Recently, circulating hematopoietic stem cells were shown to differentiate into vascular cells that participate in atherosclerosis.25,26 Furthermore, progenitor cells, including VEGFR2+ cells, have been identified in the adventitia of murine and human arteries.25,26 These progenitor cells migrate to the intima, where they can differentiate into vascular SMCs and thus promote atherosclerosis. In the present study, we confirmed the presence of these VEGFR2+ cells, which were not associated with neovessels, in the adventitia of atherosclerosis affected arteries. Although their presence in the adventitia does not prove their role in atherosclerosis as progenitor cells in our models for atherosclerosis, this study shows that VEGFR2+ cells are at least associated with atherosclerosis. In addition, VEGFR2+ cells, isolated from embryonic stem cells, have been indicated as vascular progenitor cells that can differentiate into both endothelial and smooth muscle cells.9 A recent study confirms that, although not under atherosclerotic conditions, VEGFR2+ cells are able to differentiate into SMCs in vivo during vascular recovery after denudation.10 Taking together, vaccination against VEGFR2 may have resulted in reduced amounts of progenitor cells that contribute to atherosclerosis.

The fact that VEGFR2 is highly expressed on activated endothelial cells, which cover the atherosclerotic plaque, may indicate that vaccination against VEGFR2 removes these activated cells,8 which then become replaced by “healthy” new endothelial cells. Furthermore, VEGF has been shown to accelerate atherosclerosis in hypercholesterolemic mice and rabbits.11 Although it is still not clear what the relative contribution is of the 2 major receptors for VEGF, namely VEGFR1 or VEGFR2, to atherosclerosis development, immunomodulatory capacities of VEGFR2 support their important role in lesion formation.12 Binding of VEGF to VEGFR2 induces NF-κB activation and elevated expression levels of VCAM-1, ICAM-1, and E-selectin in endothelial cells leading to increased adherence of monocytes12 and overexpression of VEGF-A in atherosclerotic lesions enhances monocyte influx and plaque expansion,27 indicating that interference in the binding of VEGF to VEGFR2 by removing VEGFR2+ cells may lead to reduced atherosclerosis. The fact that we did not observe changes in the circulating levels of proinflammatory cytokine IFN-γ suggests that vaccination against VEGFR2 does not affect atherosclerosis via interference in the systemic inflammatory response. Furthermore, VEGFR2 has recently been identified as part of a shear stress–sensing complex on endothelial cells, which becomes activated during disturbed flow patterns, which is the case at atherosclerosis-prone/affected sites of the vasculature, leading to NF-κB activation.28

Further immunohistochemical analysis of the composition of atherosclerotic plaques in the brachiocephalic and carotid arteries did not reveal any differences in the percentage macrophage, SMC, collagen, or IFN-γ staining, indicating that the inflammatory status and stability of the lesions is not affected by VEGFR2 vaccination. The finding that the neointima did contain macrophages and SMCs but no VEGFR2+ cells indicates that macrophages and SMCs are not the direct target in the vaccination against VEGFR2.

To investigate the effect of vaccination against VEGFR2 on postinterventional neointima formation, a mouse model of the common carotid artery guide wire denudation was used, which shows characteristics of a restenotic process. Shortly after denudation endothelial replacement was significantly reduced in mice that were vaccinated against VEGFR2. This delayed replacement is likely to be caused by the specific attack of proliferating endothelial cells which overexpress VEGFR2 by the CD8+ T cells. Furthermore, reduced levels of circulating endothelial progenitor cells may have contributed to delayed endothelial replacement, which is in line with a recent study in which VEGFR2+ cells in the peripheral blood have been shown to contribute to reendothelialization of balloon injured femoral arteries of mice.10 Already 1 week after denudation vaccination against VEGFR2 led to an increase in media area, and 5 weeks after denudation neointima formation in VEGFR2 vaccinated mice was 2.7-fold increased. Most likely, this increase in postinterventional neointima formation on vaccination against VEGFR2 is caused by the prolonged activation of the media, exposed to the blood stream and adhesive platelets, attributable to the delayed reendothelialization.

Very recently, the group of Curtiss30 elegantly confirmed our initial data, presented at the scientific sessions of the American Heart Association30 concerning the effect of VEGFR2 vaccination on atherosclerosis. Petrovan et al further underline the antiatherosclerotic effect of VEGFR2 vaccination by quantifying the initiation of atherosclerosis at another site in the vasculature, namely the aortic root, and also show that adventitial angiogenesis is reduced.29 Our data extend these studies and we show that also plaque progression is affected by VEGFR2 vaccination. In addition, we evaluated the presence of atherosclerosis-associated VEGFR2+ (progenitor) cells and determined the effect of vaccination on media thickness. The reduced adventitial angiogenesis in the aortic root on vaccination against VEGFR2 nicely extrapolates the previously observed effects on angiogenesis at other sites to the adventitia13,30 and
supports the hypothesis that the effect on atherosclerosis may be mediated by interference in adventitial angiogenesis. In addition we extended these studies by showing that vaccination against VEGFR2 negatively affects postinterventional neointima formation presumably by delaying endothelial replacement.

Recently, several vaccination strategies revealed promising effects on atherosclerosis. Vaccination against modified LDL, apoB100, cholesterol ester transfer protein (CETP), and interleukin (IL) 12 resulted in reduced atherosclerosis by inducing antibodies against these factors.\(^3\)–\(^3\)\(^4\) In contrast to the induction of humoral responses by vaccination against soluble proteins such as IL-12, which significantly downregulates the induction of humoral responses by vaccination against soluble proteins such as IL-12, which significantly downregulates atherosclerosis,\(^3\) this study shows that vaccination induced cell-mediated immunity against cells that potentially contribute to atherosclerosis results in reduced lesion formation. These results suggest that specific targeting of cells that contribute to atherosclerosis may form a new lead for the development of immunotherapies against atherosclerosis. The relatively low costs and the long duration of the therapeutic efficacy, which was also showed for this vaccination strategy (>10 months after the last administration of the vaccine),\(^1\)\(^3\) make vaccination an attractive tool in establishing protection against atherosclerosis. The only known adverse effect of vaccination against VEGFR2 comprises a slightly but significantly delayed wound healing (13.3 versus 14.7 days closure time of wounds of 3 mm in diameter). On the other hand, body weight, fertility, neuromuscular performance, and hematopoiesis are not affected by vaccination against VEGFR2.\(^1\)\(^3\) However, because vaccination against VEGFR2 not only results in attenuated atherosclerosis but at the same time increases postinterventional neointima formation, vaccination against VEGFR2 overexpressing cells may only be suitable for a limited patient population that does not qualify for transluminal angioplasty.

In summary, our study demonstrates that vaccination against VEGFR2 induces cell-mediated immunity against VEGFR2 leading to strongly attenuated atherosclerosis, but enhanced postinterventional neointima formation in hypercholesterolemic mice, which suggests an important role of VEGFR2 cells in atherosclerosis and restenosis. These results indicate that cell-based therapies can be of potential use in the treatment of vascular disorders and that induction of cell-mediated immunity by vaccination can be a useful tool in targeting specific atheroma-associated cell types to reduce atherosclerosis.

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Disclosures

None.

References


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A. Hauer et al.

Methods

Vaccination strategy

Briefly, pcDNA3.1-VEGFR2 was generated by cloning the mouse VEGFR2 DNA sequence with the primers 3'-CCGGTACCATGGAGAGCAAGGGCTG-5' and 5'-CCTCTAGACAGCAGCACCTCTC-3' after which this VEGFR2 DNA sequence was inserted into the pcDNA3.1 vector between the restriction sites KpnI and XbaI. Upon electroporation of pcDNA3.1-VEGFR2 or pcDNA 3.1 into S. typhimurium and selection of the plasmid containing ampicillin resistant bacteria on agar plates, the bacteria were cultured in LB medium supplemented with ampicillin (50 µg/ml). Mice were immunized by 3 times oral administration (after over night fasting and after 10 min gastric pH neutralization with 100 µl bicarbonate buffer) with 2-week intervals with 1x10^8 S. typhimurium bacteria transformed with pcDNA3.1-VEGFR2 or pcDNA3.1 (control) in 100 µl PBS, as described previously (16).

Induction of VEGFR2 specific cytotoxic CD8+ T lymphocytes

VEGFR2 expressing cells were obtained by culturing the murine fibroblast (3T3) cell line in a 96 wells plate (5.0x103 cells/well) with DMEM containing 10% Fetal Bovine Serum (FBS), 2 mmol/L L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (BioWhittaker) in a humidified atmosphere (5% CO2) at 37°C. Cells were transfected with pcDNA3.1-VEGFR2 using transfection reagent ExGen 500 (Fermentas) according
to manufacturer’s instructions. After 5 hours of transfection, cells were washed with medium and incubated with DMEM o/n. Twentyfour hours after the start of transfection 3T3 cells were loaded with 51Cr. Two hours later the loaded 51Cr cells were incubated with splenocytes from either vaccinated or control mice for 4 hrs in various splenocyte/target cell ratios. The degree of splenocyte induced cell death was measured by quantifying the amount of released 51Cr in the supernatant using a gamma counter. In order to show the important role of CD8+ T cells in the cytotoxic response, we also performed this 51Cr release assay with only CD8+ T cells as effector cells (effector/target cell ratio of 50:1), which we isolated from the total spleen cell population using CD8 specific magnetic beads (BD, Biosciences).

In a separate experiment we exposed pcDNA3.1-VEGFR2/pDNA3.1-GFP co-transfected 3T3 cells (cultured on cover slips in 24 wells plate) 24 hours after the start of transfection to splenocytes from either vaccinated or control mice for another 24 hours in an effector/target ratio of 50:1. Specific lysis of VEGFR2 expressing cells was analyzed by counting the GFP-positive cells with a Leica DM-RE microscope (Leica Imaging Systems). The degree of specific lysis was expressed as a percentage of GFP-positive cells that survived, where the situation in which no splenocytes were added represents 100% survival.

The potential induction of anti-VEGFR2 IgG antibodies was evaluated by measuring the amount of circulating serum antibodies specific for VEGFR2 using an immunoassay in which the extracellular domain of mouse VEGFR2 (R&D Systems) served as the antigen (coated on 96 wells plate) and in which the appropriate positive and negative controls were included.

**Angiogenesis in a hind limb ischemia model**

All animal work was carried out in compliance with guidelines issued by the Dutch government. For evaluation of anti-angiogenic effects induced by vaccination against
VEGFR2, apoE−/− mice were vaccinated as described (VEGFR2 vaccinated n=6, control vaccinated n=6) (16). One week after the last oral administration of the vaccine mice were anesthetized by subcutaneous injection of ketamine (160 mg/kg, Eurovet), xylazine (20 mg/kg, Bayer), and atropinesulfate (0.20 mg/kg, Centrafarm). Unilateral hind limb ischemia was introduced by coagulation of the left femoral artery proximal to the bifurcation of deep and superficial femoral artery, as previously described (17). Two weeks later, mice were sacrificed and left gastrocnemius muscles were dissected for immunohistochemical analysis of ischemia induced angiogenesis.

**Induction of post-interventional neointima formation**

One or 5 weeks after arterial injury mice were sacrificed and media area or neointimal lesions were analyzed in the common carotid artery. Maximal media area, developed within 1 week, was analyzed at sites more than 600 µm proximal from the carotid bifurcation. The site of maximal stenosis more than 600 µm proximal from the carotid bifurcation was used for morphometric analysis of neointimal lesions 5 weeks after denudation.

**Tissue harvesting**

Arteries and muscles were isolated after in situ perfusion for 15 minutes using PBS and subsequent perfusion using formalin for 30 minutes. Fixated tissues were embedded in OCT compound (Sakura Finetek), snap-frozen in liquid nitrogen and stored at -20°C until further use. Transverse 5 µm cryosections of arteries and muscles were prepared and were mounted on a parallel series of slides (18).

**Histology and immunohistochemistry**

Cryosections of arteries were routinely stained with hematoxylin (Sigma Diagnostics) and eosin (Merck Diagnostica). Staining of sections for SMCs was performed with an anti-alpha SMC actin antibody (clone 1A4, dilution 1:50; Sigma) combined with goat anti-mouse IgG peroxidase conjugate as secondary antibody (dilution 1:100, Nordic).
Staining for macrophages was performed as previously described (18). Sections of muscles or arteries were stained for CD31 with a rat anti-mouse antibody (clone MEC13.3, dilution 1:50, Pharmingen), for VEGFR2 with a rat anti-mouse antibody (clone Avas 12α1, dilution 1:50, Pharmingen), and for IFN-γ with a rat anti-mouse antibody (clone XMG1.2, dilution 1:50, Pharmingen) combined with a biotinylated polyclonal anti-rat secondary antibody (dilution 1:100). For visualization the signal was amplified by the CSA system (Dako) using 3,3'-diamino-benzidine as enzyme substrate for horse radish peroxidase. Sections were stained for collagen by Picrosirius Red (Direct 80) staining. For quantification of atherosclerosis in the brachiocephalic artery transverse cryosections of 5 µm were prepared in a proximal direction from the bifurcation with the right common carotid artery and right subclavian artery to the aortic arch and mounted in order on a parallel series of slides. Neointima area was calculated from haematoxylin/eosin sections by subtracting the patent lumen area from the area circumscribed by the internal elastic lamina. The maximal neointima area was considered as a representation of the amount of atherosclerosis. Quantification of atherosclerotic lesion area in the carotid artery was performed, as previously described (18). Analysis of stained sections was performed with a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems).

**Cholesterol and triglyceride assay**

Plasma cholesterol and triglyceride levels were quantified colorimetrically by enzymatic procedures using Precipath (Boehringer) as internal standard.
Online results:
A trend in decreased media size, which is defined as the area between the internal and external elastic lamina, was observed six weeks after collar placement in mice vaccinated against VEGFR2 as compared to control vaccinated mice (45,292±6,480 vs 31,715±1,573 µm², p=0.056). At 7 days after collar placement quantification of media size revealed a significantly reduced media area upon vaccination against VEGFR2 as compared to controls (Fig 5A-C; 43,750±4,040 vs 32,960±2,430 µm², p<0.05), while lesions were absent at this time. Immunohistochemical evaluation of the composition of the atherosclerotic lesions in brachiocephalic and carotid arteries did not reveal significant differences in macrophage, SMC, collagen, or IFN-γ content (percentage positive staining within the neointima at the site of maximal stenosis) between vaccinated and control mice. Furthermore, six weeks after vaccination against VEGFR2 serum IFN-γ levels did not differ between control and vaccinated mice in the collar induced atherosclerosis experiment (3.3±0.3 vs 3.1±0.1 ng/ml).
Online Figure I. Vaccination against VEGFR2 leads to significantly decreased media area (A,C) 1 week after collar placement as compared to control vaccination (B), which indicates that vaccination against VEGFR2 interferes in atherosclerosis in a very early stage. Scale bars represent 100 µm. *P<0.05.
Online Figure II. Endothelial replacement and media area after denudation of carotid arteries.

Endothelial cells were quantified by counting all flat shaped haematoxylin positive nuclei (arrows) in CD31+ cells lining the vessel wall (A). Panel B illustrates a part of the vessel wall which has not yet been covered by endothelial cells one week after denudation. Quantification of endothelial cells lining the vessel wall revealed significantly decreased endothelial replacement upon vaccination against VEGFR2 (C). Already 1 week after denudation media area was significantly enlarged upon vaccination against VEGFR2 (D). *P<0.05.
Online figure III. Effect of vaccination against VEGFR2 on neointima formation after vascular injury by denudation. Five weeks after denudation mice were sacrificed and neointima formation was analyzed in the carotid artery, which revealed a smooth muscle cell rich lesion, as determined with an antibody staining against alpha SMC actin (A). Quantification of lesions five weeks after denudation revealed a 2.7-fold increase in neointima formation (B) upon vaccination against VEGFR2 (D) as compared to control vaccination (C). **P<0.01.