DNA Vaccination Against VEGF Receptor 2 Reduces Atherosclerosis in LDL Receptor–Deficient Mice

Ramona J. Petrovan, Charles D. Kaplan, Ralph A. Reisfeld, Linda K. Curtiss

Objective—Similarities between neovascular ingrowth in atherosclerotic plaques and angiogenesis in tumors suggest that antiangiogenic factors that target tumor expansion may prove efficacious in the treatment of atherosclerosis. This study examined whether an oral DNA vaccine against the murine VEGF receptor 2 (Flk-1) with demonstrated antitumor effect through inhibition of pathological neovascularization can prevent or retard progression of atherosclerosis in hyperlipidemic low density lipoprotein receptor–deficient (LDLr−/−) mice.

Methods and Results—Vaccination against Flk-1 resulted in T cell activation, suppression of neoangiogenesis, and a marked reduction in atherosclerosis which was independent of hypercholesterolemia in both male and female mice. Immunohistochemical characterization of aortic sinus lesions showed that the decreased lesion area was not associated with reduced plaque stability and had a lower density of microvessels.

Conclusions—These findings demonstrate for the first time that a DNA vaccine targeting activated endothelial cells in atherosclerotic lesions provides direct atheroprotective effects. (Arterioscler Thromb Vasc Biol. 2007;27:1095-1100.)

Key Words: atherosclerosis ■ vaccination ■ plaque ■ endothelium ■ neovessels

Atherosclerosis, the primary cause of heart disease and stroke, is a progressive inflammatory disease of the vessel wall, elicted at sites of lipoprotein accumulation and hemodynamic turbulence. Endothelial activation supports recruitment of leukocytes and plays a central role in the development and progression of the atherosclerotic plaque and its clinical complications. In normal arteries the microvasculature is confined to the adventitia and outer media, but a common feature of advanced human atherosclerotic lesions is intimal and subendothelial neovascularization. Plaque vessels promote lipid deposition and the recruitment of inflammatory cells into the lesion, which is expected to result in lesion growth and a less stable plaque. Long-term treatment of hypercholesterolemic apoE−/− mice with inhibitors of angiogenesis reduces intimal neovascularization, the abundance of lesion macrophages, and plaque growth, suggesting a correlation between plaque angiogenesis, disease progression, and inflammatory cells in atherosclerotic lesions. Despite growing evidence of an association between neovascularization and atherosclerosis, the role of intraleisonal angiogenesis in the biology of cardiovascular disease, as well as the use of antiangiogenic therapies as atheroprotective strategy, remain controversial (reviewed in).

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Plaque vascularization is driven by angiogenic growth factors and cytokines, which stimulate endothelial cell migration and proliferation. In particular, the interaction between VEGF and VEGF receptor 2 (KDR, human; Flk-1, mouse) is key to pathologic angiogenesis and has been implicated in the development of atherosclerotic lesions. KDR is strongly expressed both on endothelial cells during angiogenesis and on the luminal endothelium of human atherosclerotic vessels, but not in normal arteries or veins.

In the present study, we hypothesized that a therapy that targets activated endothelial cells may have powerful antiatherosclerotic effects. An oral Flk-1–based DNA vaccine controls tumor growth and metastasis in mice by suppressing angiogenesis through a CD8+ T cell–mediated response against endothelial cells overexpressing Flk-1. This vaccination does not affect fertility, hematopoiesis, and neuromuscular performance, and wound healing is only slightly delayed. Vaccination is an especially attractive concept for treatment of atherosclerosis because it is expected to lead to long-lasting protective responses against this chronic disease. Here, we analyzed the effect of T cell–directed immunization with the Flk-1 vaccine on lesion development in a model of diet-induced atherosclerosis using LDL receptor–deficient (LDLr−/−) mice.

Materials and Methods

Mice and Experimental Protocol

LDLr−/− mice in a C57Bl/6 background were purchased from Jackson Laboratories and bred in-house. In 3 independent experimental studies, 2 with male and 1 with female mice, LDLr−/− mice...
8 to 10 weeks of age were orally vaccinated 3 times at 2-week intervals by gavage of 1×10⁴ CFU Salmonella typhimurium carrying either the pcDNA3.1 empty vector (Invitrogen) or a pcDNA3.1-Flk1 construct. In each study, T cell activation and antiangiogenic effects were evaluated in dedicated mice (n=8/group), 2 to 4 weeks after the last vaccination. In all other mice, atherosclerosis was initiated 2 weeks after the final vaccination by feeding the mice (n=14 to 16/group) a proatherogenic high-fat diet (HFD) containing 1.25% cholesterol, 15.8% fat, and no cholate (#94059, Harlan Teklad) for a total of 16 weeks, after which atherosclerosis was assessed (supplemental Figure I). All mouse experiments were performed according to the NIH Guides for the Care and Use of Laboratory Animals and all protocols were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

**Bacterial Strains and Cell Lines**
The murine MS1 endothelial cell line was purchased from American Type Culture Collection. Double attenuated S typhimurium (AroA-, dam-) strain RE88, kindly provided by Remedyne Corporation, was transformed with the plasmids by electroporation, as previously described.

**Analysis of Atherosclerotic Lesions**
The extent of atherosclerosis was quantified for each mouse in lipid stained en face preparations of the aorta and in cross sections of the aortic sinus, as described. Histological analysis was performed on subsequent aortic sinus sections from 4 mice per group in each experimental study, ie, 12 sections per treatment group, selected to be representative of the group average lesion size. Masson trichrome stain was used to identify collagen within the aortic sinus sections. Macrophage infiltration was visualized with rat anti-mouse MOMA-2 antibody (Serotec), biotinylated secondary antibody, and streptavidin-peroxidase, followed by color reaction with AEC (Vector Labs), and counterstaining with hematoxylin. The percent of lesion area stained blue (trichrome) and red (MOMA-2), respectively, was calculated by digital image analysis.

**Evaluation of Antiangiogenic Effects**
Microvessel staining was performed with rat anti-mouse CD31 (clone MEC 13.3; BD Pharmingen), followed by Alexa 647 goat anti-rat IgG (Invitrogen) and nuclear staining with DAPI. Samples were viewed using a Rainbow Radiance 2100 laser scanning confocal system with a Nikon TE2000-U inverted microscope (Bio-Rad–Zeiss). Images were analyzed for quantitative fluorescence using Image Pro Plus 3DS (Media Cybernetics) and Image J (NIH Imaging) software. The total area occupied by vessels was assessed by quantifying the CD31+ area in and around each of the 3 valve cusps individually.

**Statistical Analysis**
All results were expressed as mean±SD, except the lesion data where individual values are shown. Statistical difference of extent of atherosclerosis between experimental groups and controls was analyzed using the nonparametric Mann–Whitney rank sum test in the Statview SE+ statistics package (SAS Institute Inc). All other analyses used a two-tailed, unpaired t test.

**Results**

**Vaccination of LDLr⁻/⁻ Mice Against Flk-1 Leads to Suppression of Angiogenesis and a Specific T Cell Response**
Suppression of angiogenesis after Flk-1 vaccination was demonstrated qualitatively and quantitatively by performing Matrigel assays. Macroscopic analysis revealed a marked reduction in vascularization of the Matrigel plugs after vaccination against Flk-1 (Figure 1A). In vivo staining of the endothelium with fluorescein-labeled Isolectin B4 showed that vessel number and size were decreased after vaccination with the vector encoding Flk-1 compared with control mice (Figure 1B). This difference in blood vessel formation was further demonstrated quantitatively, as i.v. injection of Evans blue revealed lower dye content in Matrigel plugs harvested 2 to 3 weeks after the last immunization and subsequent culture for 5 days, as previously described.

**Cytokine Release and Cytotoxicity Assays**
Enzyme-linked immunosopot (ELISPOT) assays were performed to measure single-cell cytokine release. Splenocytes, collected 2 to 4 weeks after the last immunization, were cultured overnight with murine endothelial MS1 cells and assayed in triplicates with an IFN-γ-ELISPOT kit (BD Biosciences). MS1 murine endothelial cells expressing full-length Flk-1, or the synthetic FLK400 peptide (Multiple Peptide Systems) in the presence or absence of an inhibitory rat anti-mouse monoclonal antibody to CD8 (clone 2.43; Biovest Inter-
against Flk-1 induced a CD8+ cells was demonstrated with a standard 51Cr release assay. Addition of an inhibitory monoclonal antibody to CD8 before stimulation specifically blocked the IFN-γ production of IFN-γ was increased in splenocytes from mice immunized with the Flk-1 vaccine when compared with splenocytes from control mice. Addition of an inhibitory monoclonal antibody to CD8 before stimulation specifically blocked the IFN-γ secretion (Figure 2A), indicating the involvement of CD8+ T cells.

Antigen-specific cytotoxicity against Flk-1–positive MS1 cells was demonstrated with a standard 51Cr release assay. Immunizations with the vector encoding Flk-1 led to increased lysis of murine endothelial target cells expressing Flk-1 when compared with control immunized animals (Figure 2B). No measurable cytotoxicity was detected against target cells not expressing Flk-1 (not shown). Taken together these results indicate that vaccination of LDLr−/− mice against Flk-1 induced a CD8+ T cell–mediated response that suppressed angiogenesis.

Plasma Cholesterol Levels and Distribution Are Not Affected by Vaccination

Two weeks after the final vaccination, mice were fed a proatherogenic high-fat, high-cholesterol diet (HFD) for a total of 16 weeks. All mice displayed normal growth rates throughout the study (not shown). As expected, plasma cholesterol levels were greatly elevated in all mice fed the HFD, with significantly higher levels in male mice as compared with their female counterparts at all time points. Importantly, there was no difference in total cholesterol levels between the Flk-1 and empty vector vaccinated mice in any study, indicating that the Flk-1 vaccine had no impact on diet-induced hypercholesterolemia (supplemental Table I). In addition, a similar distribution of lipoprotein cholesterol was found in samples from mice vaccinated with either the Flk-1 construct or the empty vector control, both before and after high-fat feeding, indicating that immunization did not affect cholesterol distribution. (supplemental Figure II).

Levels of Circulating Flk-1 Are Lower in Vaccinated Mice

To further examine the effect of vaccination against Flk-1 in LDLr−/− mice, levels of soluble Flk-1 (sFlk-1) were assessed in pooled plasma samples from each study group using a mouse sFlk-1 ELISA. Recombinant forms of sFlk-1 bind to VEGF and mediate anti-tumor effects. More recently, a naturally occurring, truncated form of Flk-1 was detected in mouse and human plasma. This sFlk-1, which is secreted or proteolytically cleaved from endothelial cells, retains ligand binding and may therefore play a role in VEGF-mediated biological functions. Two weeks after the last immunization, plasma levels of sFlk-1 were similar in nonvaccinated, empty vector–treated or Flk-1–vaccinated mice, indicating that immunization did not affect circulating Flk-1 (Figure 3). Although the Flk-1 content in human atherosclerotic vessels is consistent with the expected vaccine-induced destruction of plasminogen activator inhibitors, this result is consistent with the expected vaccine-induced destruction of
those endothelial cells that are overexpressing Flk-1 in hyperlipidemic LDLr<sup>−/−</sup> mice.

**Vaccination Decreases Atherosclerotic Lesion Size and Microvessel Density Without Plaque Destabilization**

Progression of atherosclerotic lesions differs between male and female mice and varies slightly between studies performed at different times. Therefore, atherosclerosis was assessed in 3 independent experimental studies, 2 with male and 1 with female mice, 16 weeks after initiation of the HFD, ie, at week 22 of the study. Despite similar total plasma cholesterol levels, formation of aortic atherosclerotic lesions was substantially decreased in mice vaccinated with the Flk-1 construct compared with controls (Figure 4). Although female LDLr<sup>−/−</sup> mice developed more extensive aortic sinus lesions than their male counterparts, similar differences in lesion development compared with controls were observed in all three studies, regardless of lesion size. En face lipid staining of the entire length of the aorta as well as analysis of aortic sinus lesion area showed a significant reduction in lesion size after vaccination against Flk-1 in each of the 3 studies analyzed individually (Figure 4) or when data were pooled for each vaccine (not shown). These findings demonstrate that a prophylactic vaccination against proliferating endothelial cells overexpressing Flk-1 can attenuate progression of atherosclerosis in high fat fed LDLr<sup>−/−</sup> mice.

To determine the effect of vaccination on lesion morphology, aortic sinus lesion areas comprising the entire intima, including lipid cores and fibrotic components, were examined. Histological characterization was performed on sections selected based on their size (nearest to the mean lesion area of each study group) to facilitate a comparison between lesions formed in Flk-1 vaccinated and control mice. As shown in Figure 5A, smaller and fewer lipid cores were present in lesions from mice which received the Flk-1 vaccine as compared with control vaccinated mice. Masson trichrome staining revealed a more uniform collagen matrix in mice vaccinated against Flk-1, but lesional collagen levels were comparable between both male and female mice (not shown). When normalized to percentage of total lesion area, there were no detectable effects of vaccination on collagen content in aortic sinus sections from all mice. MOMA-2 macrophage-specific immunostaining showed that immunization against Flk-1 had no impact on macrophage content of the lesions in any of the study groups, as the mean percentage of all sinus lesions that stained positively for MOMA-2 was comparable (Figure 5A and 5B). Thus, immunohistochemical analysis revealed no changes in plaque stability.

To examine the effect of vaccination on microvessel density in and around the atherosclerotic lesions, CD31-stained sections were evaluated by confocal microscopy. In the control mice, the presence of microvessels around the lesion was obvious, whereas in the mice vaccinated against Flk-1 only a few neovessels were detected (Figure 5C). A quantitative analysis showed that CD31-positive areas in the Flk-1 immunized mice were ≈2.4-fold lower compared with the control mice (Figure 5D). Taken together, these results suggest that Flk-1–based vaccination against activated endothelial cells may lead to a less advanced lesion phenotype.

**Discussion**

The present report demonstrates an atheroprotective effect achieved by immunization with a DNA vaccine against the murine VEGF receptor 2, Flk-1. DNA vaccines encoding highly immunogenic viral or bacterial antigens can elicit a strong humoral response. In contrast, relatively weak self-antigens like Flk-1 in the mouse are not capable of producing a measurable antibody response in syngeneic mice (R.A. Reisfeld, unpublished data), but induce a moderate CD8<sup>+</sup> and/or CD4<sup>+</sup> T cell response. Our results show that vaccination of LDLr-deficient C57BL/6J mice against Flk-1 did induce activation of CD8<sup>+</sup> T cells and antigen-specific T cell–mediated cytotoxicity against murine endothelial cells (Figure 2) and provide evidence that targeting atherosclerosis through genetic immunization against activated endothelial cells overexpressing Flk-1 translates into diminished growth of plaques in high fat fed LDLr<sup>−/−</sup> mice (Figure 4). This result is consistent with the concept that proliferating endothelial cells functionally contribute to active plaque angiogenesis and atherosclerotic lesion progression. However, the spe-
specific mechanisms by which DNA vaccination against Flk-1 reduces atherosclerosis remain to be established.

Intraplaque neovascularization has been identified as a critical factor in lesion stability and plaque rupture, but the consequences of blocking plaque angiogenesis on the inflammatory component of atherosclerosis and on plaque stability are not fully understood. Interestingly, an earlier report demonstrated that in apoE/H/H mice, antibody-blocking of Flk-1 is ineffective against development of neovasculature in lesions or in surrounding adventitia and does not affect progression of atherosclerotic plaques. These results appear to contradict our data, but blockade of receptor signaling by antibody and T cell-mediated removal of receptor-expressing proliferating endothelial cells in different mouse models of experimental atheroma are unlikely to have comparable effects. Our vaccination against Flk-1 quantitatively reduced plaque development but this does not exclude the possibility that it might also cause adverse qualitative changes in plaque morphology, such as increased accumulation of infiltrated leukocytes and release of matrix metalloproteinases that could lead to plaque destabilization. However, our immunohistochemical analysis of representative aortic sinus sections from three independent studies indicated that DNA vaccination against Flk-1 attenuated atherosclerotic lesion development in LDLr/H mice without reducing plaque stability.

A recent report indicated a correlation between plaque progression, vasa vasorum (VV) neovascularization, and adventitial inflammation in hyperlipidemic apoE/H/LDLr/H double knockout mice. These mice display severe lesions in the aorta with considerable structural similarity to human atherosclerosis and develop intraplaque vessels that communicate with adventitial VV. We did not assess the microvessel density along the aorta of the vaccinated LDLr/H mice, but found that neovascularization in atherosclerotic regions of the aortic sinus in mice vaccinated against Flk-1 was considerably decreased compared with control mice. If intimal neovascularization is indeed a precondition for advanced atheroma development, suppression of initial angiogenesis through removal of proliferating endothelial cells expressing Flk-1 could have led to reduced plaque formation. However, atherosclerotic lesions in the aortic root are initiated at earlier stages of the disease and progress faster than in the aorta, and therefore lesion growth in LDLr/H mice might not directly depend on ongoing neovascularization within our experimental time frame.

It is widely accepted that there is an enhanced production and release of inflammatory mediators in atherosclerotic lesions and that these mediators not only act to elicit and sustain the local intramural inflammatory response, but also can enter the circulation and induce a systemic inflammatory response. Although our evaluation of lesion morphology revealed no difference in macrophage infiltration relative to total lesion area, it is possible that lesion-specific inflammatory changes induced by the Flk-1 vaccine were primary mediators of the attenuated atherosclerosis development. Moreover, vaccination against Flk-1 could have led to the
removal of activated endothelial cells at atherosclerotic lesions, thereby eliminating the major promoters of vascular inflammation and thrombosis, which in turn resulted in atheroprotection. Alternatively, alterations in other Flk–mediated biological functions of VEGF and/or of the Flk-1–dependent processes in vascular endothelium in response to shear stress may also have contributed to the beneficial effect of the vaccination against Flk-1.

Traditional cardiovascular intervention modalities aimed at attenuation of atherogenesis have focused on the reduction of risk factors, but very few modalities are concerned with the beneficial effect of the vaccination against Flk-1.

DNA vaccine may represent an additional choice for the treatment of atherosclerosis. Our findings provide the first evidence that the Flk-1–based DNA vaccine may represent an additional choice for the treatment of atherosclerosis.

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Disclosures

None.

References

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Data Supplement

Table I
Plasma cholesterol levels were not affected by vaccination

<table>
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<th>Vaccine</th>
<th>Week</th>
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<th>Study #2 (n =14/group)</th>
<th>Study #3 (n ≥15/group)</th>
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<tr>
<td>Control</td>
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<td>336.62 ± 48.08</td>
<td>232.78 ± 27.12</td>
<td>195.40 ± 21.32</td>
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<td>1729.76 ± 341.27</td>
<td>1255.58 ± 261.82</td>
<td>1082.63 ± 129.82</td>
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<td>1357.05 ± 308.22</td>
<td>1443.55 ± 301.63</td>
<td>1055.05 ± 116.72</td>
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<tr>
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<td>1373.55 ± 182.04</td>
<td>1471.23 ± 348.47</td>
<td>1195.94 ± 297.87</td>
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<tr>
<td>Flk-1</td>
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<td>250.92 ± 23.27</td>
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<tr>
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<td>1564.53 ± 301.20</td>
<td>1444.17 ± 297.29</td>
<td>1176.67 ± 223.15</td>
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</tbody>
</table>

Figure I. **Detailed experimental protocol.** The experimental design of each of the three independent studies utilized groups of LDLr-/- mice (n=15) at 8-10 weeks of age, with males and females studied separately. An additional 8 mice per group were included in each study to evaluate self antigen-specific immunity and suppression of angiogenesis following immunization. Mice were fasted overnight, weighed and venous blood was drawn from the retro-orbital sinus into a heparinized capillary tubes at four weeks intervals. Total plasma cholesterol levels were measured in individual samples by a colorimetric assay (Sigma). For the sFlk-1 ELISA (R&D Systems) and FPLC fractionation, equal volumes of samples were pooled from all mice of each experimental group for each time point. Plasma samples from groups of non-vaccinated mice from parallel studies were included as a control for vaccination.

Figure II. **Plasma lipoprotein distribution.** Distribution of total cholesterol within the major lipoprotein fractions was analyzed by fast-performance liquid chromatography (FPLC) in pooled plasma samples obtained from each study group (n ≥14 mice) before
the initiation of the HFD and at the end of the experimental period. Plasma samples from a group of non-vaccinated mice were included as a control for vaccination. The vast majority of total plasma cholesterol was redistributed to the VLDL/LDL lipoprotein fractions after high fat feeding, which showed a ≈10-fold higher level compared to levels before consumption of the HFD. a) FPLC profile showing cholesterol concentration in each fraction before (top) and after (bottom) HFD in samples from male (closed symbols) and female (open symbols) mice. Normalized FPLC profiles b) before initiation of HFD and c) at the end of the experimental period demonstrate similar distribution of cholesterol to lipoproteins in samples from male (bottom) and female (top) mice. To facilitate comparisons between groups, cholesterol in each fraction is expressed as a percentage of the total cholesterol recovered from the column.
Blood samples at 4 week intervals

Angiogenesis & T-cell Assays

Vaccinate

HFD

Assessment of Atherosclerosis

Figure I
Figure II

(a) Plasma Cholesterol (mg/dl)

(b) Plasma Cholesterol (% of Total)

(c) Plasma Cholesterol (% of Total)