Immunization of apoE−/− mice with aldehyde-modified fibronectin inhibits the development of atherosclerosis

Pontus Dunér1*, Fong To1, Karsten Beckmann2†, Harry Björkbacka1, Gunilla Nordin Fredrikson1,3, Jan Nilsson1, and Eva Bengtsson1

1Department of Clinical Sciences, Skåne University Hospital, Lund University, Malmö, Sweden; 2Department of Molecular Medicine, Max Planck Institute of Biochemistry, 82152 Martinsried, Germany; and 3Department of Biomedical Laboratory Science and Technology, Malmö University, Malmö, Sweden

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Aims

Oxidation of low-density lipoprotein (LDL) in the extracellular matrix of the arterial wall results in the formation of malondialdehyde (MDA) that modifies surrounding matrix proteins. This is associated with the activation of an immune response against modified extracellular matrix proteins present in atherosclerotic plaques. Clinical studies have revealed an inverse association between antibodies to MDA-modified fibronectin and risk for development of cardiovascular events. To determine the functional role of these immune responses in atherosclerosis, we performed studies in which apoE-deficient mice were immunized with MDA-modified fibronectin.

Methods and results

Immunization of apoE-deficient mice with MDA-modified fibronectin resulted in a 70% decrease in plaque area and a less inflammatory phenotype of remaining plaques. Immunization shifted a weak naturally occurring Th1 antibody response against MDA–fibronectin into a Th2 antibody response. Cytokine expression and flow cytometry analyses of spleen cells from immunized mice showed an activation of regulatory T cells. Immunization with MDA–fibronectin was also found to reduce plasma fibronectin levels.

Conclusion

Immunization with MDA–fibronectin significantly reduces the development of atherosclerosis in apoE-deficient mice suggesting that the immune response observed in humans may have a protective effect. MDA–fibronectin represents a possible novel target for immunomodulatory therapy in atherosclerosis.

Keywords

Atherosclerosis • Immunization • ApoE−/− mice • MDA–fibronectin

1. Introduction

Retention of low-density lipoprotein (LDL) by the extracellular matrix in the arterial wall and subsequent LDL oxidation are key steps in atherosclerotic disease. The retention of LDL involves interaction of apoB-100 protein in LDL with extracellular matrix proteoglycans. Mice expressing LDL-binding-defective proteoglycan develop less atherosclerosis.1,2 In addition, other extracellular matrix proteins such as collagen, laminin, and fibronectin have been shown to bind lipoproteins.3,4 LDL oxidation is associated with the decomposition of fatty acids, resulting in the formation of reactive aldehydes, such as malondialdehyde (MDA) and 4-hydroxynonenenal, binding covalently to amino groups in the apoB-100 protein. These oxidized LDL neoantigens are targeted by the immune system, and epidemiological studies have demonstrated associations between autoantibodies against oxidized LDL antigens and cardiovascular disease.5–7

A possibility that has been largely unexplored is that reactive aldehydes released during LDL oxidation results in MDA modifications also of the surrounding extracellular matrix proteins. These modified matrix proteins could potentially target immune responses against the extracellular matrix of the plaque. Fibronectin is an extracellular matrix protein, which is up-regulated in atherosclerotic lesions5 and, interestingly, deposited at atherosclerosis-prone sites before others signs of atherosclerosis in mice.9 Using an in vitro model, we have recently found that LDL oxidation results in MDA modifications of fibronectin and that MDA-modified fibronectin is present in...
atherosclerotic lesions. In addition, antibodies against MDA-modified fibronectin were associated with cardiovascular disease in a prospective case–control study. However, in contrast with our expectations, baseline autoantibody levels were found to be significantly lower in subjects that later suffered a fatal or non-fatal myocardial infarction than in controls, suggesting that these autoimmune responses have a protective effect. To determine the functional role of these antibodies in atherosclerosis, we immunized apoE-deficient mice with MDA-modified fibronectin.

2. Methods

2.1 Materials

Rat fibronectin was purchased from Biomedical Technologies Inc., and mouse fibronectin from Innovative Research. Recombinant protein fragments spanning fibronectin’s type III domains 1–5, 4–7, 7–10, 10–12, 12–14, and 13–15 were expressed and purified as previously described. 1, 11, 13, 3, 3-tetraethoxy propane (Sigma) was used for chemical MDA modification.

2.2 Animals

Six-week-old male C57BL/6 apoE<sup>−/−</sup> mice (Taconic, Denmark) were immunized with 85 µg rat MDA-modified or native fibronectin (97% identity to mouse fibronectin) or phosphate-buffered saline (PBS) using Alum (Pierce) as adjuvant, followed by two booster injections after 3 and 5 weeks. The mice were fed a high-fat diet, 21% cocoa fat, 0.15% cholesterol (Pierce) as adjuvant, followed by two booster injections after 3 and 5 weeks. One control group followed the diet programme without getting injections. Tissue was preserved as described previously. 12 All animal experiments were approved by the Malmö-Lund Animal Care and Use Committee, and the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Plasma cholesterol was quantified colourimetrically using Infinity Cholesterol (ThermoTrace).

2.3 Immunohistochemistry

Preparation, staining, and quantifications in the descending aorta or in sub-valvular plaques were performed as previously described. 12 Briefly, lipids were stained in en face preparations of the descending aorta with Oil Red O. Subvalvular plaques were stained with MOMA-2 antibodies (BMA Biomedicals, Switzerland) recognizing monocytes/macrophages, with affinity-purified biotinylated anti-mouse IgG (Vector Laboratories), with neutrophil antibody NIMP-R14 (abcam, 2557), with polyclonal rabbit anti-murine fibronectin antibodies (abcam, ab23750), or with IgG against MDA–fibronectin produced in rabbit, 10 using rabbit immunoglobulins (DAKO) as negative control and counterstained with haematoxylin. The specificity of rabbit anti-MDA–fibronectin for MDA-mouse fibronectin, but not native mouse fibronectin was verified by enzyme-linked immunosorbent assay (ELISA). Polyclonal anti-murine fibronectin antibodies were recognized both native and MDA-modified fibronectin.

2.4 Splenocyte proliferation and cytokine production

Mice immunized with MDA–fibronectin with Alum, native fibronectin with Alum, PBS with Alum, or left non-treated as described earlier were killed at 12 weeks of age and spleens were harvested. Splenocytes were collected by mechanical force through a 70 µm cell strainer (BD Falcon), and erythrocytes were removed using red blood cell lysing buffer (Sigma). The cells were cultured in culture media (RPMI 1640 media containing 10% heat-inactivated foetal calf serum, 1 mmol L<sup>−1</sup> sodium pyruvate, 10 mmol L<sup>−1</sup> Hepes, 50 U penicillin, 50 µg mL<sup>−1</sup> streptomycin, 0.05 mmol L<sup>−1</sup> mercaptoethanol, and 2 mmol L<sup>−1</sup> l-glutamine, Gibco) in 96-well round bottom suspension cell plates (Sarstedt). Splenocytes were cultured with Concanavalin A (Con A, Sigma), 30 µg mL<sup>−1</sup> rat MDA–fibronectin, 30 µg mL<sup>−1</sup> rat native fibronectin, or with medium alone. CD28 monoclonal antibody (17 µg mL<sup>−1</sup>; BD Pharmingen) was added to wells with unstimulated splenocytes, and cells stimulated with native or MDA–fibronectin in the cytokine assays. Splenocytes were incubated for 72 h, after which cytokine assay plates were terminated, and proliferation plates were pulsed with 1 µCi [methyl-<sup>3</sup>H] thymidine (Amershann) for further 20 h. Cells were then harvested (Packard) onto glass fibre filter (Wallac), and thymidine incorporation was measured using a liquid scintillation counter (Wallac). The cytokine release from cultured splenocytes was analysed using a transforming growth factor-β (TGF-β) ELISA kit (R&D Systems) or a multiplex kit measuring interleukin-1β (IL-1β), IL-2, IL-4, IL-5, IL-10, IL-12, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and keratinocyte chemoattractant (KC) (Meso Scale Discovery, USA). IL-1β values in measured samples were below the detection limit.

2.5 ELISA

Proteins were either MDA-modified on the microtitre plate or in solution using 0.05 mol L<sup>−1</sup> MDA in PBS pH 7.4. MDA modifications were assayed using thiobarbituric acid reactive substances assay. 11 ELISA, for measuring antibody response and determining immunoglobulin isotype in immunized mice, was performed using microtitre plates coated with 10 µg mL<sup>−1</sup> native or MDA-modified mouse fibronectin, and plasma pooled from five or eight mice in each group or from individual mice was added. Bound antibodies were detected by biotinylated anti-mouse IgG or IgM produced in goat (Jackson ImmunoResearch), followed by alkaline-phosphatase-conjugated streptavidin, or with alkaline-phosphatase-conjugated anti-mouse-IgG1 produced in rat (BD Pharmingen), or alkaline-phosphatase-conjugated IgG2a produced in rat (BD Pharmingen). Observations that were 1.5 times the inter-quartile range beyond the appropriate quartile were regarded as outliers and excluded from the analysis. Fibronectin in plasma from mice was analysed with AssayMax mouse fibronectin ELISA kit (DayMoon Industries Inc.).

2.6 Statistical analysis

Statistical analyses were performed with SPSS version 12.0.1. Values are presented as mean ± s.d. or as individual values. We used ANOVA or Kruskal–Wallis followed by post hoc test to compare multiple groups. For skewed variables, the non-parametric Mann–Whitney test was used for comparisons of data.

Additional methods are presented in Supplementary material online, Methods.

3. Results

3.1 Immunization of apoE<sup>−/−</sup> mice with MDA–fibronectin protects against atherosclerosis

To study the functional role of antibodies against MDA–fibronectin in atherosclerosis, we immunized 6-week-old apoE<sup>−/−</sup> mice with 85 µg rat MDA-modified fibronectin using Alum as adjuvant. Mice immunized with native fibronectin, adjuvant alone, and non-immunized were included as controls. Booster immunizations were given 3 and 5 weeks later. The mice were killed at 26 weeks of age, and the severity of atherosclerosis was assessed by en face Oil Red O staining of the aorta and by the measurement of plaque area of subvalvular lesions. Immunization with MDA–fibronectin was found to reduce atherosclerosis in the aorta by 60% (1.0 ± 0.6% lesion area of total
vessel area) when compared with animals injected with adjuvant alone (2.6 ± 2.1%) and by 69% when compared with mice given no treatment (3.3 ± 1.2%; Figure 1A). The plaque area of subvalvular lesions was decreased by 27% (2.6 ± 0.6 x 10^2 μm^2) in MDA–fibronectin-immunized mice when compared with the adjuvant control (3.6 ± 1.1 x 10^2 μm^2; Figure 1B) and mean plaque area demonstrating positive immunoreactivity for macrophages was reduced from 8.2 ± 2.8 x 10^3 μm^2 in the adjuvant control mice to 6.0 ± 1.4 x 10^3 μm^2 (mean ± s.d.; P < 0.05) in immunized mice (Figure 1D). In contrast, there was a trend towards increased T-cell accumulation (P = 0.06) in the lesions of MDA– fibronectin-immunized mice (Figure 1C). Mice immunized with native fibronectin also had reduced atherosclerosis in aorta (0.9 ± 0.5%) and decreased subvalvular lesions (2.0 ± 0.7 x 10^3 μm^2) compared with adjuvant-treated or non-immunized mice (Figure 1A and B). Moreover, macrophage immunoreactivity was reduced in plaques of native fibronectin-immunized mice (4.7 ± 1.0 x 10^3 μm^2, P < 0.05; Figure 1D), and there was also a trend towards increased T-cell accumulation (Figure 1C). There was no significant difference in neutrophil content between the groups (see Supplementary material online, Figure S1). Histological examinations did not reveal any vascular alterations outside atherosclerotic lesions in response to immunization with native or MDA–fibronectin.

The observation that immunization with native fibronectin inhibited atherosclerosis was unexpected. However, since it has been shown that aluminium potently induces oxidative stress and lipid peroxidation, 13–17 we explored the possibility that immunization with fibronectin together with Alum as adjuvant would result in MDA modifications of the injected native fibronectin. Therefore, mice were immunized as mentioned already, and tissue from the injection site was homogenized and analyzed by fluorescence measurements of aldehyde groups (Figure 2). The analysis revealed the presence of MDA-modified protein at the injection site of mice immunized with native fibronectin. Accordingly, it is likely that immunization with native fibronectin mimics that of immunization with MDA–fibronectin, and it is even possible that this immune response more resembles that induced by MDA modification of fibronectin in vivo.

3.2 Immunization of apoE−/− mice results in a Th2-type antibody response

Immunization of apoE−/− mice with either MDA-modified or native fibronectin resulted in high levels of IgG binding MDA–fibronectin (Figure 3A). ELISA studies demonstrated that IgG antibodies binding to MDA–fibronectin could be competed by addition of MDA–fibronectin (data not shown). The antibodies were almost exclusively of the IgG1 isotype, reflecting activation of a Th2-type immune response (Figure 3B). Non-treated mice or mice treated with adjuvant alone had only very low levels of IgG against MDA–fibronectin, and these autoantibodies were primarily of the IgG2a isotype indicative of a weak, naturally occurring Th1 response against MDA–fibronectin (Figure 3B). To study whether the antibodies were recognizing site-specific epitopes in the fibronectin protein, we tested the antibody reactivity to various MDA-modified fragments of fibronectin, which comprise the fibronectin type III domain containing part of the protein (Figure 3C). The results demonstrated that the antibody response was not restricted to a single site but occurred at multiple sites along the entire sequence of the protein.

To analyse whether circulating antibodies bind to MDA–fibronectin antigen in the vessel wall, we stained subvalvular lesions from MDA–fibronectin-immunized mice with anti-IgG and anti-MDA–fibronectin. Although antibodies recognizing IgG showed a more widespread distribution than anti-MDA–fibronectin, the result clearly shows colocalization of MDA–fibronectin and IgG, indicating that antibodies from the plasma bind MDA–fibronectin (see Supplementary material online, Figure S2).

Immunized mice were also found to develop antibodies recognizing native fibronectin as assessed by ELISA. To determine whether these antibodies cross-reacted with native fibronectin also in vivo, we analysed the effect of immunization on plasma fibronectin levels. Indeed, circulating fibronectin levels in 26-week-old mice were found to be significantly reduced in both mice immunized with MDA-modified (640 ± 90 μg mL⁻¹) and native fibronectin (630 ± 160 μg mL⁻¹) when compared with adjuvant (840 ± 180 μg mL⁻¹) or non-immunized mice (920 ± 120 μg mL⁻¹; Figure 4). The decrease in circulating fibronectin suggests the possibility that the protective effect of immunization could be due to cross-reactivity of antibodies with the plasma protein resulting in reduced accumulation of fibronectin in plaque tissue. However, immunohistochemical analysis did not demonstrate any significant difference in native or MDA-modified fibronectin in plaques (see Supplementary material online, Figure S3). We also analysed subvalvular sections from 12-week-old mice, but found only occasional and small lesions. However, in such lesions, MDA–fibronectin immunoreactivity was frequently present (data not shown).

3.3 Immunization of apoE−/− mice results in a regulatory T-cell response

To determine whether the atheroprotective effect of MDA–fibronectin immunization was associated with changes in antigen-specific cellular immune responses, we isolated splenocytes from 12-week-old non-immunized, PBS/adjuvant, native, or MDA–fibronectin-immunized mice. Splenocytes from MDA–fibronectin-immunized mice were characterized by an increased proliferation in response to stimulation with MDA–fibronectin or native fibronectin, the latter indicating that cellular tolerance to the native protein was broken (Figure 5A). Exposure of spleen cells from MDA–fibronectin-immunized mice to the polyclonal activator Con A resulted in lower proliferation when compared with splenocytes isolated from non-immunized mice or mice given adjuvant alone (Figure 5A). To characterize the presence of regulatory T cells in immunized mice, we measured FoxP3, CD4, and CD25-positive cells by flow cytometry (Figure 5B). Increased regulatory T cells were seen in both MDA-modified and native fibronectin-immunized mice. The relative presence of these cells was not further increased by stimulation with MDA-modified protein (data not shown). To analyse whether there was an increase in cytokines associated to regulatory T cells, we measured TGF-β and IL-10 secretion from cultured splenocytes of immunized mice. An increased secretion of TGF-β was observed in cultured splenocytes from both Alum (501 ± 70 pg/500 000 cells) and MDA–fibronectin-immunized mice (520 ± 82 pg/500 000 cells) compared with non-immunized mice (286 ± 53 pg/500 000 cells; P < 0.0001). The secretion of TGF-β was not further enhanced by exposing the cells to MDA–fibronectin (data not shown). Splenocytes from both native and MDA–fibronectin-immunized mice were also characterized by an increased antigen-dependent secretion of IL-10 and IFN-γ (Figure 6A and B).
Figure 1 Immunization of apoE−/− mice with MDA–fibronectin reduces atherosclerosis. (A) En face preparations of the descending aorta of 26-week-old non-immunized mice (non-imm), alum-immunized mice (Alum), native fibronectin (natFN), or MDA–fibronectin (MDA–FN)-immunized mice were stained with Oil Red O. (B and C) Subvalvular lesions from 26-week-old-immunized mice were stained with haematoxylin or anti-CD3. (A and B) Areas were quantified by computer-aided morphometry. (D) Subvalvular lesions from 26-week-old-immunized mice were stained with antibodies recognizing monocytes/macrophages and counterstained with haematoxylin. Scale bar = 500 μm. **p < 0.005, *p < 0.05, a p = 0.06, b P = 0.08.
The present study identified several potential mechanisms through which MDA-fibronectin immunization may protect against atherosclerosis, including modulation of the Th1/Th2 balance, activation of regulatory T cells, and cross-reactivity with native fibronectin resulting in lower levels of plasma fibronectin, and lower cholesterol levels. Thus, our study provides important clues, which may help to explain the association between autoantibodies against MDA–fibronectin and lower cardiovascular risk observed in humans.

Immunizations with native and MDA–fibronectin were unexpectedly found to be equally effective in inhibiting the development of atherosclerosis. However, an analysis of tissue extracted from the injection site from mice immunized with native fibronectin revealed the presence of MDA-modified fibronectin, indicating that modifications of the protein may take place after immunization. This is in accordance with previous studies showing that aluminium induces oxidative stress and lipid peroxidation.13–17 The presence of antibodies reacting with MDA-modified fibronectin in native fibronectin-immunized mice further supported the notion that modification of the protein takes place locally at the injection site. Accordingly, the atheroprotective effect of immunization with native fibronectin most likely involves activation of an immune response against MDA–fibronectin.

Interestingly, immunization with MDA–fibronectin was also associated with activation of several subtypes of immunosuppressive regulatory T cells (Treg), including FoxP3-expressing natural Tregs, TGF-β-producing Th3, and IL-10-producing Tr1 cells.18 All of these types of Tregs have previously been shown to reduce the development of atherosclerosis.19–21 In addition, stimulation of splenocytes from MDA–fibronectin-immunized mice with the polyclonal activator Con A resulted in decreased proliferation when compared with the effect on cells from non-immunized or adjuvant-treated mice, supporting the notion that immunization with MDA–fibronectin induced a functional suppression through activation of regulatory T cells. Accordingly, two effects on regulatory T cells were induced by immunization with MDA–fibronectin. The first was Alum-dependent, involving activation of TGF-β-producing Th3 cells and may explain the trend towards an atheroprotective effect observed in the mice treated with adjuvant alone. The second was observed only in mice immunized with native or MDA–fibronectin and involved activation of FoxP3-positive natural Tregs and IL-10-producing Tr1 cells. Both natural Treg and Tr1 cells have been demonstrated to have protective roles in atherogenesis in several mouse models. Treatment of apoE−/− mice with CD25-antibody depleting natural Treg cells resulted in a 50% increase in lesion size when compared with animals given control IgG.19 Transfer experiments using IL-10-producing Tr1 cells to apoE−/− mice have been shown to reduce atherosclerosis.22 In addition, LDLr−/− mice with leucocyte IL-10 deficiency developed markedly increased atherosclerosis.23 Thus, adjuvant alone induces a regulatory T-cell response, which is further enforced by immunization with native or MDA–fibronectin-activating antigen-specific natural Treg and Tr1 cells. Immunization with both native and MDA–fibronectin also induces a pro-inflammatory IFN-γ response, but this is counteracted by an anti-inflammatory IL-10 response. Both the anti-inflammatory IL-10 and the pro-inflammatory IFN-γ...
responses were antigen-specific and activated by both native and MDA-modified fibronectin. It seems likely that MDA–fibronectin is formed upon immunization in native fibronectin-immunized mice, explaining the immune responses against MDA–fibronectin in these mice. However, the immune reactions against the native protein in both groups indicate that tolerance against the native protein is broken.

It is conceivable that the reverse association between MDA–fibronectin autoantibodies and cardiovascular risk in humans can be explained by a similar mechanism as in the MDA–fibronectin-immunized mice. According to this theory, the autoimmune responses against MDA–fibronectin in humans would be accompanied by counteracting activation of antigen-specific Tregs, which in turn would exert plaque-stabilizing, anti-inflammatory effects in MDA–fibronectin-containing plaques.

Another possibility is that immunization with MDA–fibronectin results in the generation of antibodies that cross-react with native
fibronectin in atherosclerotic lesions and mediates the removal of the protein. The reduced plasma level of fibronectin observed in immunized mice could potentially also contribute to a reduced presence of fibronectin in plaque tissue. Fibronectin is involved in the regulation of cell adhesion, migration, and proliferation and is up-regulated in atherosclerotic lesions. Although the functional role of fibronectin in atherosclerosis remains to be fully understood, recent studies demonstrating that hypercholesterolaemic mice lacking the alternatively spliced fibronectin II domain have a retarded progression of disease support a pro-atherogenic effect. These data were confirmed by Babaev et al. who, however, also showed that mice constitutively expressing IIIA had reduced atherosclerosis, suggesting that regulation of the splicing of the extra type III domain A exon is involved in the disease. However, the finding that plaques from immunized mice did not contain less fibronectin immunoreactivity argues against the possibility that the atheroprotective effects observed in the present study are explained by immune responses against plaque fibronectin.

An additional factor that may contribute to the inhibition of atherosclerosis observed here is the decrease in plasma cholesterol levels in immunized mice. Lower plasma cholesterol levels have also been observed in fibronectin IIIA-deficient mice, further implicating fibronectin in lipoprotein metabolism. Several clinical studies have also reported associations between plasma fibronectin and cholesterol levels. The mechanisms responsible for these associations remain to be fully understood, but may involve interactions of fibronectin with lipoprotein receptors. Taken together, these observations suggest that the athero-protective effect of immunization with MDA–fibronectin in mice is in part due to cholesterol lowering in response to decreased levels of plasma fibronectin. Such a mechanism is unlikely to act in humans, since no relation between autoantibodies and plasma cholesterol levels was observed in association studies between MDA–fibronectin autoantibodies and cardiovascular risk.

**Figure 4** Immunization of apoE−/− mice with MDA–fibronectin reduces fibronectin levels in plasma. Fibronectin levels in plasma from 26-week-old non-immunized (non-imm), Alum (Alum), native fibronectin (natFN), or MDA–fibronectin (MDA–FN)-immunized mice was analysed by ELISA. **P < 0.005, *P < 0.05; ANOVA followed by post hoc test.**

**Figure 5** Immunization of apoE−/− mice with MDA–fibronectin induces antigen-specific T-cell proliferation and regulatory T cells. (A) Splenocytes from 12-week-old mice immunized with MDA–fibronectin (MDA–FN), Alum (Alum), or non-immunized control mice (non-imm; x-axis) were stimulated with 30 μg mL−1 native fibronectin, MDA–fibronectin, or Con A, and T-cell proliferation was assayed by [3H]-thymidine uptake. Proliferation index is defined as the ratio of proliferation of stimulated cells to non-stimulated cells. (B) To analyse the presence of regulatory T-cell lymphocytes from spleens of 12-week-old apoE−/− mice immunized with Alum (Alum), native fibronectin (natFN), MDA–fibronectin (MDA–FN), or non-immunized (non-imm) mice were analysed by flow cytometry to determine the percentage of CD3+/CD4+ cells expressing CD25/FoxP3. n = 11–12 (A) or n = 4–6 (B) in each group; **P < 0.05, ***P < 0.001, ****P < 0.0001; Mann–Whitney test.
Interestingly, immunization with MDA–laminin in apoE−/− mice has been found to result in increased atherosclerosis. This was accompanied by an increase in proinflammatory Th17 effector cells and a decrease in Foxp3-positive regulatory T-cells. It remains to be fully understood why immunization with MDA–laminin results in a proinflammatory immune response and increases atherosclerosis, whereas immunization with MDA–fibronectin induces an anti-atherogenic regulatory T-cell immune response. Fibronectin is present in almost every tissue and is likely to be oxidized also in other locations than the atherosclerotic lesions. It is possible that an immune response recognizing MDA–fibronectin would have more adverse effects than immune responses against MDA–laminin, which has a more restricted distribution. Therefore, it may be more critical for regulatory T-cells to control autoimmune reactions against MDA–fibronectin.

It should be kept in mind that in a human setting, this type of immunomodulatory therapy most likely would be administered when atherosclerotic lesions are already formed. In this study, immunizations of mice were started at 6 weeks of age before high-fat diet. Although cholesterol levels and lesion formation is enhanced upon high-fat diet, it is likely that LDL oxidation and MDA modification are present already at the start for immunization. ApoE−/− mice have increased lipid levels compared with wild-type mice, and develop atherosclerosis even without high-fat diet. In addition, lipid retention in the artery is present by 3 weeks of age, and monocyte attachment to endothelium is observed at 5 weeks of age on a normal chow. Interestingly, fibronectin has been shown to present in the vessel wall at a very early stage, before fatty streak development.9

In conclusion, we demonstrate that induction of an immune response against MDA–fibronectin has an atheroprotective effect, supporting the clinical observation of an inverse association between MDA–fibronectin autoantibodies and cardiovascular risk. The mechanisms involved in this protection remain to be fully elucidated, but appear to involve effects on the balance of Th1/Th2-type immunity, activation of regulatory T-cells, and a decrease in fibronectin and plasma cholesterol levels. Immune responses against aldehyde-modified fibronectin represent a novel mechanism that may be involved in modulating the atherosclerotic disease processes and suggest a new possibility of immunization strategy to reduce atherosclerosis in humans. However, it should be recognized that modulating immune responses against MDA–fibronectin in established human disease is likely to be more complex and needs further investigation.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

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