

Extracellular matrix components in atherosclerotic arteries of Apo E/LDL receptor deficient mice: An immunohistochemical study

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Summary. During accelerated vascular remodeling such as in atherosclerosis, the composition of the extracellular matrix becomes altered. The matrix components of the diseased artery influence cellular processes such as adhesion, migration and proliferation. Furthermore, in atherosclerosis, the inability of the cells within the lesion to produce a mechanically stable matrix may lead to plaque rupture. In this immunohistochemical study of atherosclerotic mice aorta, we have reviewed the presence of ECM components with roles in maintaining tissue structure and function. These components include osteopontin and COMP as well as the leucine rich repeats proteins decorin, PRELP, and fibromodulin. Immunohistochemistry demonstrated presence of osteopontin, COMP, decorin, PRELP and fibromodulin in lesion areas of ApoE/LDLr deficient mice. Some advanced lesions exhibited areas of cartilage-like morphology and were shown to represent cartilage by their content of the cartilage specific proteins collagen II and aggrecan. The results suggest that cartilage-associated cell/collagen binding ECM proteins may be involved in the pathogenesis of atherosclerosis.

Key words: Atherosclerosis, Extracellular matrix, ApoE/LDL receptor deficient mice, Leucine rich repeats proteins

Introduction

During the progression of atherosclerosis, an extensive remodeling of the extracellular matrix (ECM) takes place. The development of atherosclerosis is initiated by an inflammatory process, triggered by an increased uptake of LDL particles in the intimal space of the vessel wall. Cytokines, growth factors and adhesion molecules attract medial smooth muscle cells (SMC),

which subsequently start to proliferate and produce ECM proteins, including collagen. Growth factors and cytokines further promote growth of the plaque and formation of a fibrous cap which encapsulate the lesion. At a later stage, matrix-degrading enzymes secreted by macrophages, may weaken the fibrous cap leading to thrombus formation (Ross, 1999; Rosenfeld, 2000). A number of recent reports provide examples of how the ECM participates during different stages of the disease. An altered ECM affects cellular adhesion, migration and proliferation (Raines, 2000). It has also been shown that a modified ECM influences collagen fiber formation (Barnes and Farndale, 1999), lipid retention (Williams and Tabas, 1995) and calcification (Watson et al., 1998). Vascular calcification has been found to be an organised process with similarities to bone formation (Doherty and Detrano, 1994) and recent studies have shown that bone and cartilage-associated proteins are present in atherosclerotic lesions (Dhore et al., 2001; Canfield et al., 2002).

In this study, we have analyzed the expression of 5 cartilage-associated ECM proteins with cell and/or collagen binding properties in atherosclerotic aorta of apoE/LDLr double knockout mice. Collagen binding proteins have been shown to affect collagen fiber assembly and their presence may be of significance for the formation of functional collagen fibers, and thereby affect plaque stability. Cell-interacting ECM components may be important for SMC migration and proliferation as well as for the calcification process. We have used antibodies against osteopontin, Cartilage Oligomeric Matrix Protein (COMP), decorin, Proline, arginine-Rich End Leucine-rich repeat Protein (PRELP) and fibromodulin. Furthermore, we have analyzed the development of cartilage-like areas of arterial lesions and cartilage proteins within these areas. In this study we have used the apoE/LDLr deficient mouse, which develops severe hyperlipidaemia and atherosclerotic lesions spontaneously. This model is particularly useful for studies of ECM proteins since vascular remodeling, which involve ECM reorganisation, is part of the atherosclerotic process in these mice (Bonthon et al.,

1997).

Some of the investigated proteins (osteopontin, COMP and decorin) have been identified in human atherosclerotic lesions (O'Brien et al., 1994; Riessen et al., 1994, 2001), while others have never before been studied in vascular disease (PRELP and fibromodulin). A detailed presentation of the expression of these five ECM components is required for a better understanding of their roles during the atherosclerotic process.

Materials and methods

Animals

The study was approved by the local ethical committee and conforms to the guide for the care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). Apo E^{-/-}/LDLr^{-/-} (B6.129- Apo^e^{tm1Unc} Ldlr^{tm1Her}) and control mice with background strain (C57BL/6x129) were purchased from JAX mice, Jackson Laboratories, Bar Harbour, ME. The animals were fed regular diet and given water ad libitum. Mice were sacrificed by carbon dioxide inhalation.

Antibodies

Monoclonal α -smooth muscle actin (NH₂-peptide) antibody was purchased from Sigma, rat anti mouse macrophage/monocyte antibody (MOMA-2) from Serotec, UK and monoclonal rat osteopontin (MPIIB10-1) from Developmental Studies Bank, University of Iowa, USA. The following rabbit polyclonal antiserum described elsewhere were used against; bovine aggrecan (Murphy et al., 1999), bovine osteopontin (Franzén and Heinegård, 1985), and rat COMP (Joosten et al., 1999). The antibody against collagen II (Mo and Holmdahl, 1996) was a kind gift from professor Rickard Holmdahl, Lund University, Sweden. Polyclonal PRELP antiserum was raised against recombinant rat PRELP, expressed in 293-EBNA cells (Bengtsson et al., 2000) and affinity purified on the recombinant protein. Polyclonal antiserum was also raised against decorin and fibromodulin, purified from rat bone and human cartilage respectively.

Western blotting

Animals were sacrificed; aortas localised, surgically removed and rinsed in PBS, dissected clear and ground in liquid nitrogen. The homogenate was extracted with 4M-guanidine chloride HCl, 5mM N-ethylmaleimide, pH 5.8 on a shaking table at 4 °C for 24 hours. After centrifugation at 12000 g at 4 °C for 20 min, the supernatant was precipitated twice in 10 volumes of 96% ethanol, 50 mM sodium acetate for 8 hours. Precipitates were dissolved in electrophoresis sample buffer (2% SDS, 125 mM Tris-HCl, pH 6.8, 0.01% bromphenol blue and 20% glycerol). Total protein concentration was

determined by using DC Protein Assay (Biorad laboratories CA) according to the manufacturers protocol. Samples (15 μ g of total protein) were electrophoresed on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Hybond-C Extra, Amersham Lifescience). The membranes were blocked with 7% non fat dry milk and incubated for 1 hour with appropriate antibody. Detection was made with HRP conjugated secondary antibody followed by ECL incubation and development on film. Western blotting was repeated at least three times for each antibody.

Tissue preparation for immunohistochemistry

A cut was made in the portal vein for venous outflow. Aortas were perfused with 4% paraformaldehyde (Sigma) in phosphate buffered saline pH 7.3 by slowly injecting 10 ml of fixative to the left ventricle of the heart. The aorta was taken out and put in fixative for 24 hours. Specimens were rinsed in PBS, dehydrated in a series of increasing ethanol concentrations and embedded in paraffin (Histowax, Histolab, Gothenburg, Sweden). Sections (5 μ m) were cut with a microtome. Tissue sections were deparaffinized with xylene and rehydrated with graded ethanols.

Immunohistochemistry

Endogenous peroxidase activity was quenched by incubating the sections with 3% hydrogen peroxide in methanol for 10 min. Sections were rinsed in phosphate buffered saline (PBS) and incubated with normal goat serum (Vectastain elite kit, Vector laboratories, Inc Burlingame, CA) before the primary antibody was added. Primary antibodies were diluted in PBS/0.3% bovine serum albumin (BSA) and incubated overnight in a humidified chamber. Subsequent staining followed the protocol of the manufacturer (Vectastain Elite kit, Vector laboratories, Inc Burlingame, CA). Sections were incubated with diaminobenzidine (DAB) substrate kit (Vector laboratories, Inc Burlingame CA) for 1-5 min. After washes in PBS, sections were stained with haematoxyline for 20 s, dehydrated in ethanol/xylene and mounted with pertex (Histolab, Gothenburg, Sweden). Immunohistochemical stainings were performed at least twice with each antibody. For all the proteins studied except osteopontin, the same antibody was used in immunostainings and Western blotting. A polyclonal rat osteopontin antibody was used in immunohistochemistry and a monoclonal rat osteopontin was used in Western blotting. Specificity of the antibodies was assured by adding excess antigen to the antiserum, which diminished antibody staining in immunohistochemistry (data not shown). These controls were carried out on antibodies where corresponding antigen was available (bovine OPN, rat COMP and rat decorin). The lesion presented in figures 2 and 7 represent data from

consecutive sections.

Results

Cellular composition of the lesions

The cellular content in aortic lesions from apo E/LDLr deficient mice was analyzed with immunohistochemistry using macrophage (MOMA-2) and smooth muscle cell (α -actin) specific antibodies. Fig. 1 shows consecutive sections with positive staining for α -actin (Fig. 1A,C) and MOMA-2 (Fig. 1B,D) from two different lesions of 24 weeks old mice. Positive immunostaining for α -actin was present in the media and in the fibrous cap of the lesions (Fig. 1A,C). MOMA-2 reactivity colocalized with cells of foam cell-like morphology (Fig. 1B,D). Areas of cartilage-like morphology with fewer cells but more abundant ECM compared to other lesions was detected in advanced plaques from 24 weeks old knock-out mice. These regions were also found to contain the cartilage specific proteins collagen II (Fig. 2A) and aggrecan (Fig. 2B). They were also positive for COMP, fibromodulin and PRELP (Fig. 7), all present in normal cartilage. There was no reactivity for α -actin or for MOMA-2 (Fig.

2C,D).

Osteopontin

We demonstrate the presence of osteopontin in lesions of aortas from 20 and 24-week old knock-out mice (Fig. 3A). Areas rich in macrophages showed a more intense staining for osteopontin than those containing SMC. In normal aorta, low levels of osteopontin were found in the innermost part of the media (Fig. 3A). However, in Western blotting, presence of osteopontin was restricted to diseased aortas. A band of 65 kDa corresponding to the size of osteopontin was demonstrated in protein extract from 24 weeks old apo E/LDLr deficient mice. This band was not present in the control aortic extract (Fig. 6A).

COMP

The expression pattern for COMP in apo E/LDLr deficient mice resembles that for osteopontin. Strong staining for COMP was detected in lesions dominated by macrophages but was also found in lesions rich in SMC (Fig. 3B). COMP was also detected in small amounts in the media of normal aorta (Fig. 3B). In Western blotting,

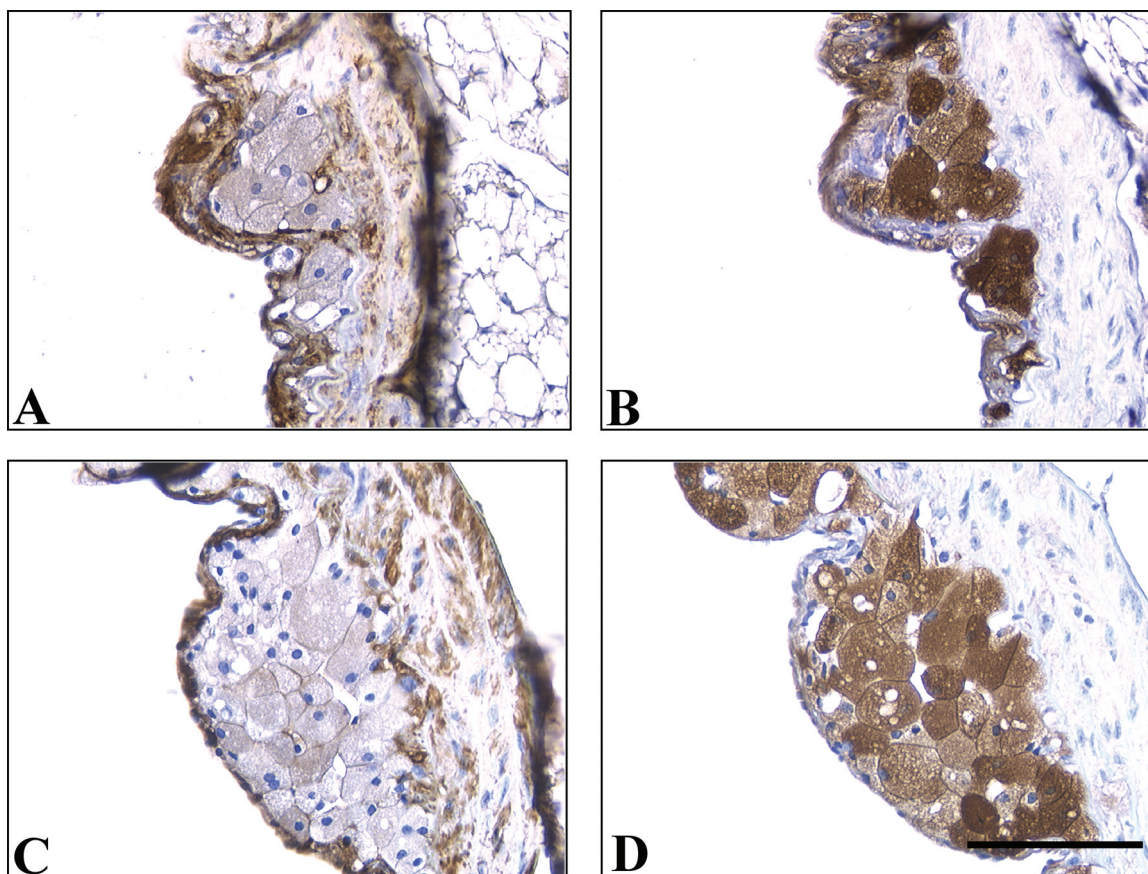


Fig. 1. Cellular composition of the lesions. Localization of SMC and macrophages in consecutive sections from lesions of 24 weeks old apoE/LDLr deficient mice. SMC was detected with SMC specific α -actin-antibody (**A and C**) and macrophages with MOMA-2 (**B and D**). Scale bar: 100 μ m.

the presence of COMP was restricted to diseased arteries. A band of approximately 100 kDa could be detected in protein extract from aortas of knock-out mice but not in aortas of control mice (Fig. 6B). COMP was also present in the cartilage-like structure of the lesion seen in Figure 7A,B, where the protein mainly localized pericellularly.

Decorin

By immunohistochemistry we found presence of decorin in atherosclerotic as well as in normal aortas (Fig. 4). However, the most abundant decorin immunoreactivity was detected in lesion areas where it colocalized with SMC. The protein was also present in the fibrous cap region (Fig. 4). In Western blotting one strong and polydisperse band (60-80 kDa) was detected in protein extracts of both atherosclerotic and normal aortas (Fig. 6C).

PRELP

PRELP was present in plaques of 20 and 24 week-old knock-out mice with a more abundant expression in

macrophage-dominated lesions compared to those rich in SMC (Fig. 5A). Low levels of PRELP staining could be found in normal aorta and in the media of diseased aortas (Fig. 5A). In Western blotting experiments, one band of about 60 kDa could be detected in the lane with extract from diseased aortas as well as in the lane with normal aortic extract (Fig. 6D). PRELP was also present in the advanced lesion with cartilage-like morphology, but restricted to a pericellular localization in specific regions of this structure (Fig. 7C,D).

Fibromodulin

We demonstrate the presence of fibromodulin in lesion areas of 20 and 24 weeks old knock-out mice (Fig. 5B). Figure 5B demonstrates abundant fibromodulin immunoreactivity, localized to areas of macrophage-like cells, in a lesion of a 20-week-old mouse. A weak fibromodulin staining was also found in normal aorta (Fig. 5B). Figure 6E shows a Western blot with bands corresponding to fibromodulin (65 kDa) in lanes with extracts from normal as well as from diseased aorta. The lesion with cartilage-like morphology expressed fibromodulin in a pericellular fashion in some

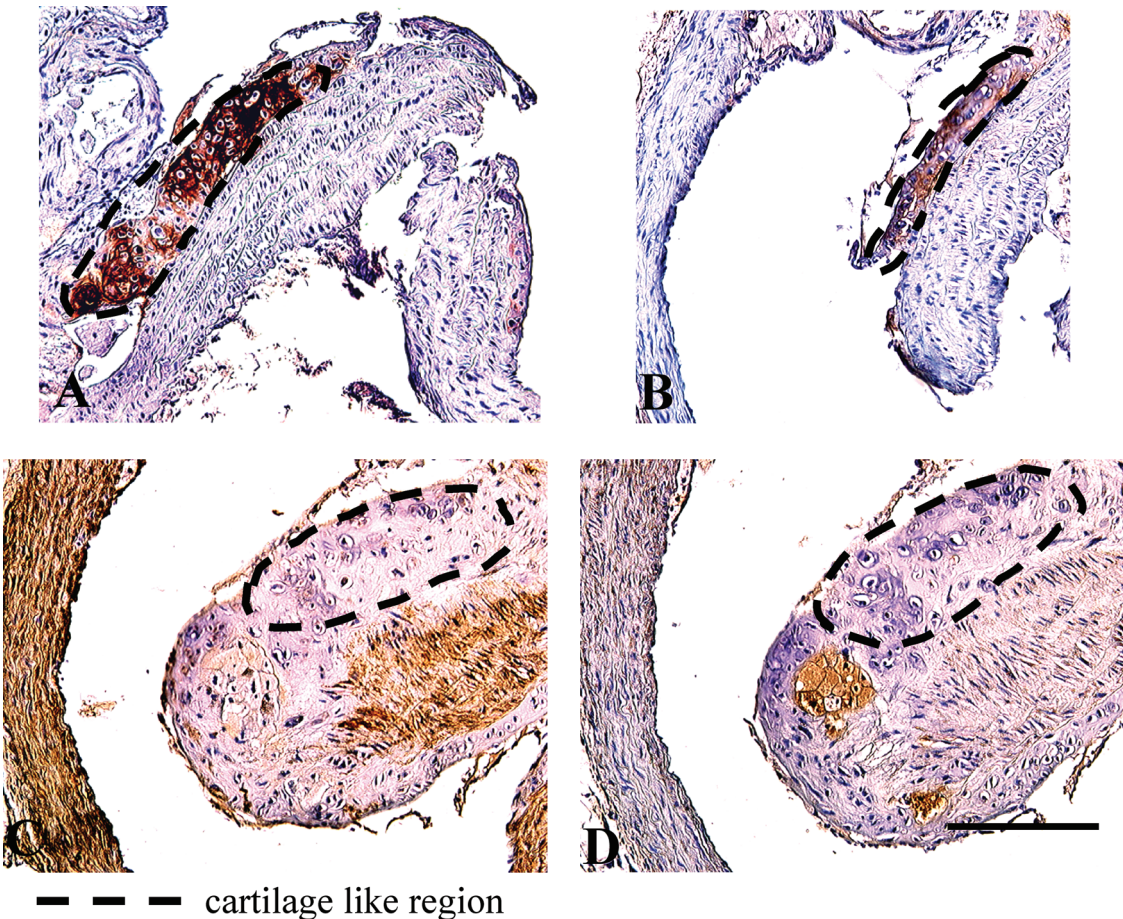


Fig. 2. Advanced lesion with cartilage structure. Detection of cartilage-specific proteins in an advanced lesion of a 24 weeks old apo E/LDLr knock-out mouse. The region with cartilage-like morphology (encircled with a dotted line) stained positive for collagen II (A) and aggrecan (B), whereas it stained negative for α -actin (C) and MOMA-2 (D). Scale bar: 100 μ m.

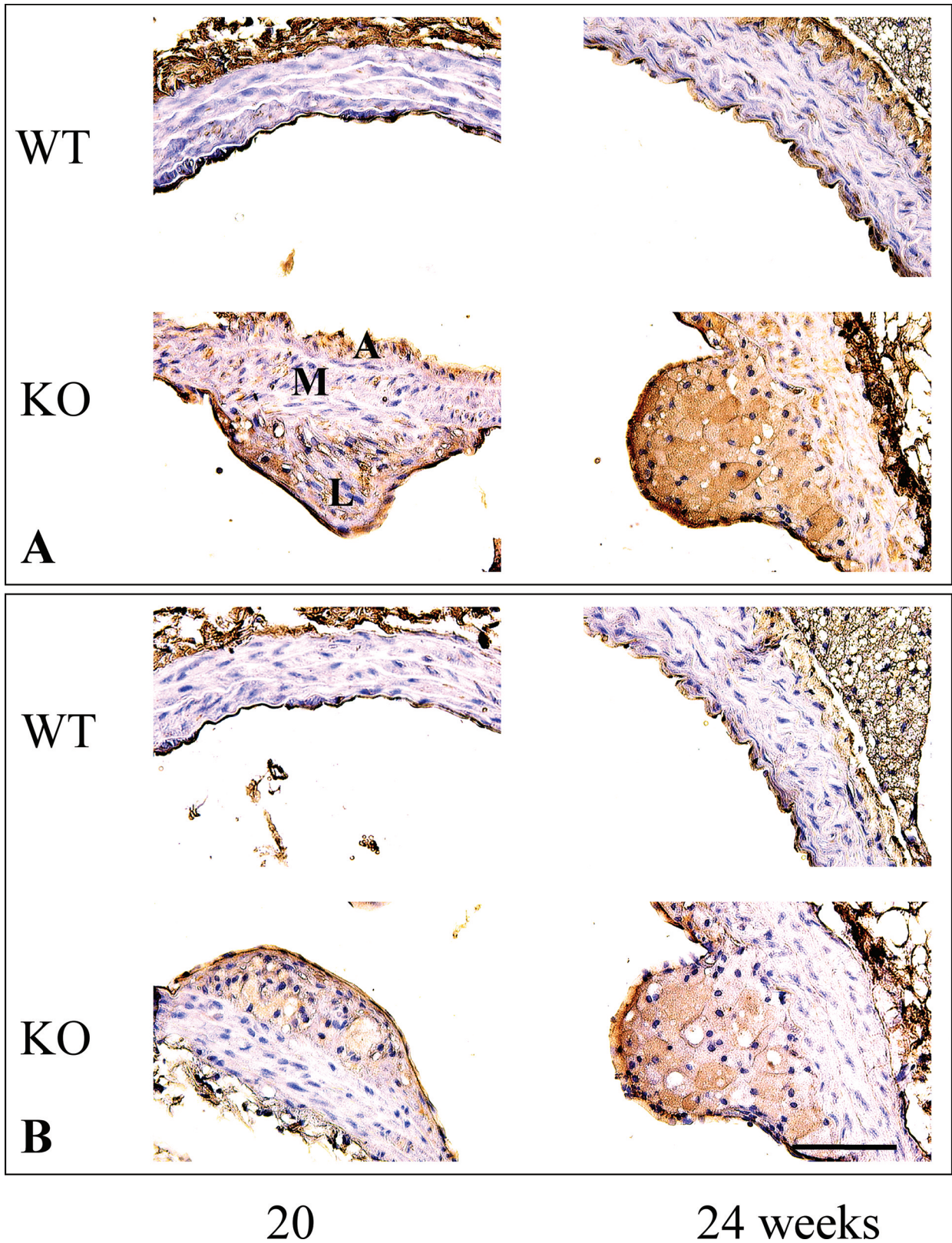


Fig. 3. Immunohistochemistry demonstrating presence of osteopontin (A) and COMP (B) in aortas of 20 and 24 weeks old apo E/LDLr deficient (KO) and wild type (WT) mice. A: adventitia, M: media, L: lesion. Scale bar: 100 μ m.

areas of this structure (Fig. 7E,F).

Discussion

The present study demonstrates the presence of several cell binding and/or collagen binding ECM proteins in atherosclerotic lesions of apo E/LDLr deficient mice. The difference in localization for the ECM proteins within the plaques in combination with their ability to either bind to cells in the vessel wall and/or take part in collagen fiber assembly reflect different functions for these proteins during the disease process.

Elevated levels of osteopontin in human atherosclerotic plaques have been described by several groups (Giachelli et al., 1993; O'Brien and Garvin, 1994). In agreement, we found osteopontin in lesions of apo E/LDLr knock-out mice. Osteopontin was detected in regions rich in macrophages with a foam cell like morphology. This finding is consistent with that of Reckless and co-workers (Reckless et al., 2001), who found osteopontin in lipid filled lesions in a number of different atherosclerotic mouse strains. Indeed, osteopontin has been shown to be synthesised by

macrophages and to modulate macrophage adhesion (Nasu et al., 1995), migration and cytokine release (Singh et al., 1990). Furthermore, there are a number of reports demonstrating the involvement of osteopontin in processes like SMC calcification (Shanahan et al., 1994; Wada et al., 1999), adhesion and migration (Liaw et al., 1994). We also found osteopontin in fibrotic lesions in association with SMC, although with a lower expression than in macrophage filled lesions. It is likely that osteopontin has a dual role in atherogenesis, acting both as a modulator of the inflammatory process and as a regulator of SMC behaviour.

COMP is present in cartilage, tendon, and synovium (Hedbom et al., 1992; Di Cesare et al., 1997). As was the case for osteopontin, COMP expression was found in lesion areas, and with a similar distribution within the plaques. A more intense staining was detected in macrophage rich regions compared to areas rich in SMC. Recently, COMP has been shown to be produced by human vascular SMC in vitro as well as being present in atherosclerotic and restenotic human arteries (Riessen and Fenchel, 2001). COMP can bind SMC (Riessen and Fenchel, 2001) and may thereby alter SMC function in the growing plaque. Furthermore, COMP has been

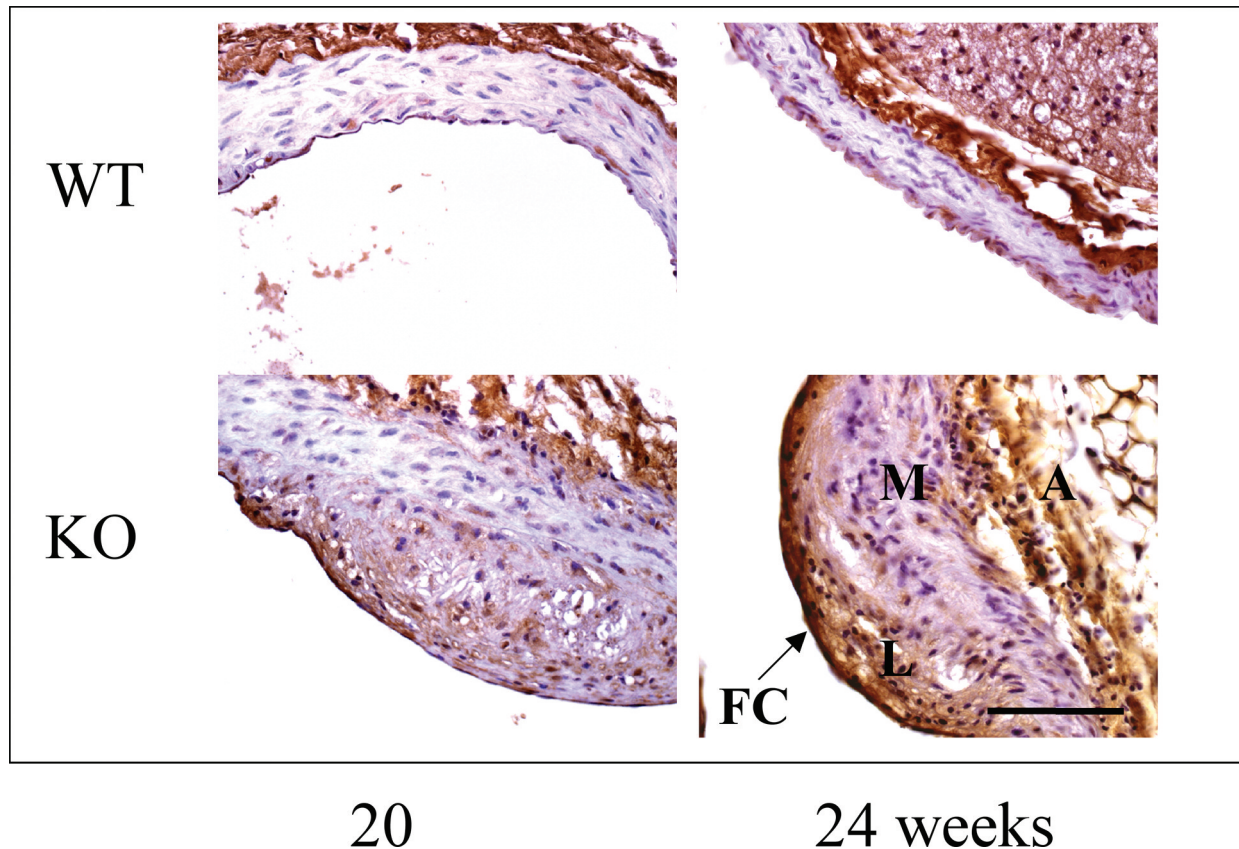


Fig. 4. Immunohistochemistry demonstrating presence of decorin in aortas of 20 and 24 weeks old apo E/LDLr deficient (KO) and wild type (WT) mice. A: adventitia, M: media, L: lesion, FC: fibrous cap. Scale bar: 100 μ m.

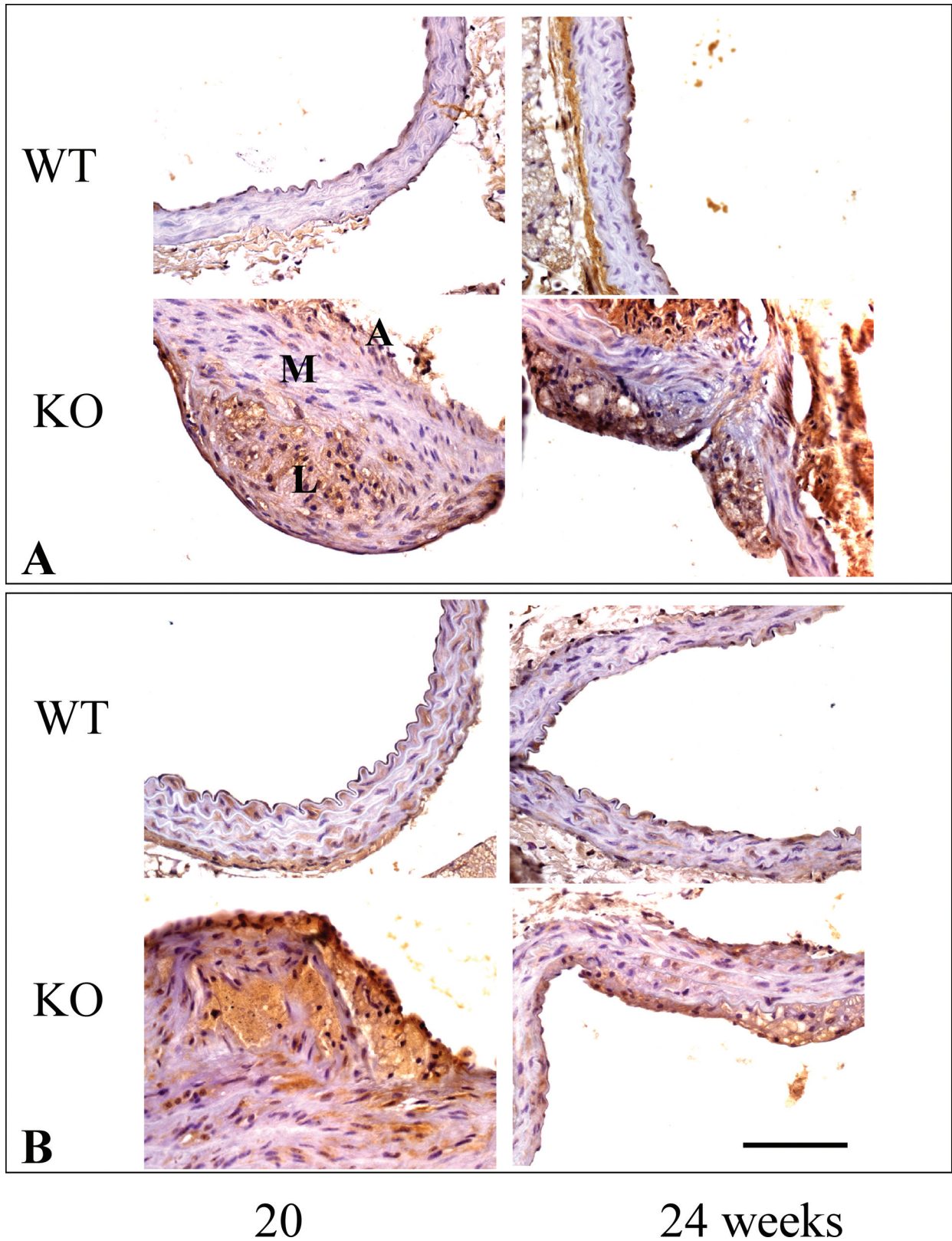


Fig. 5. Immunohistochemistry demonstrating presence of PRELP (A) and fibromodulin (B) in aortas of 20 and 24 weeks old apo E/LDLr deficient (KO) and wild type (WT) mice. A: adventitia; M: media, L: lesion. Scale bar: 100 μ m.

proposed to play a role as regulator of collagen fiber formation (Rosenberg et al., 1998), and may as such also be important for the collagen assembly during lesion

formation.

The leucine rich repeat (LRR) ECM proteins form a family of proteins with a structure suitable for

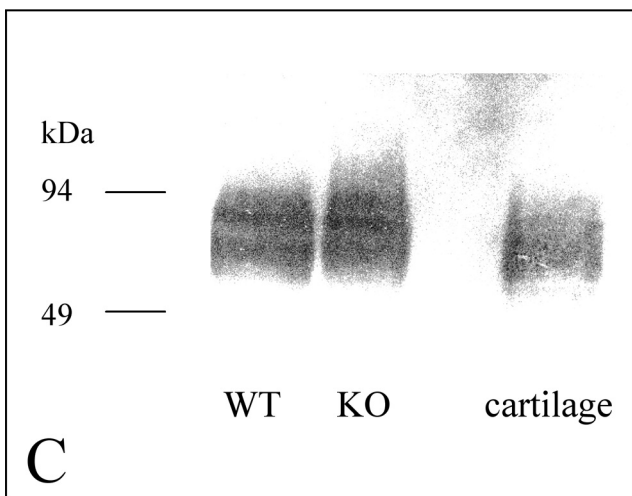
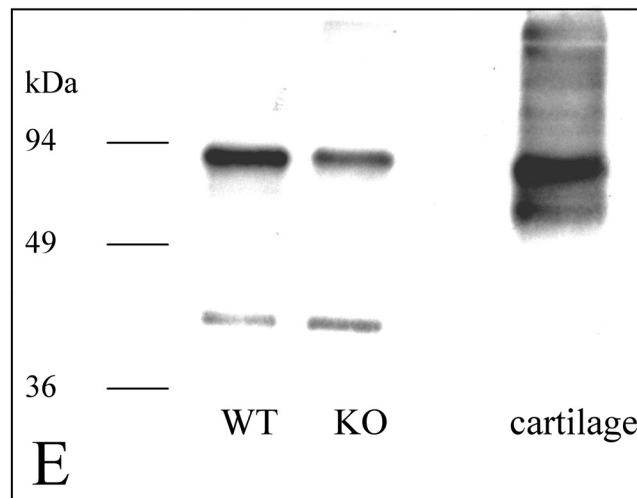
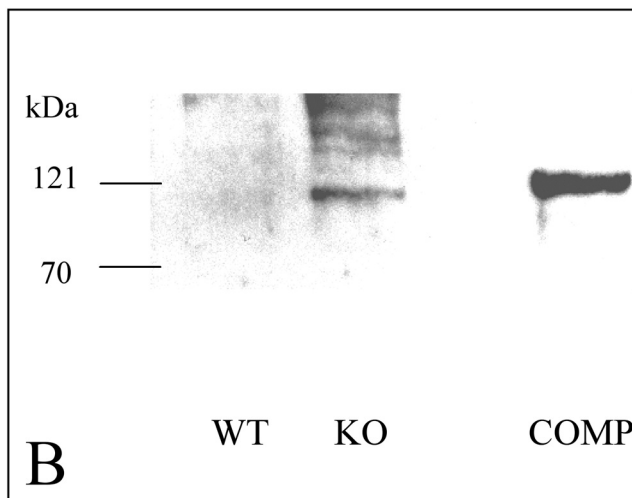
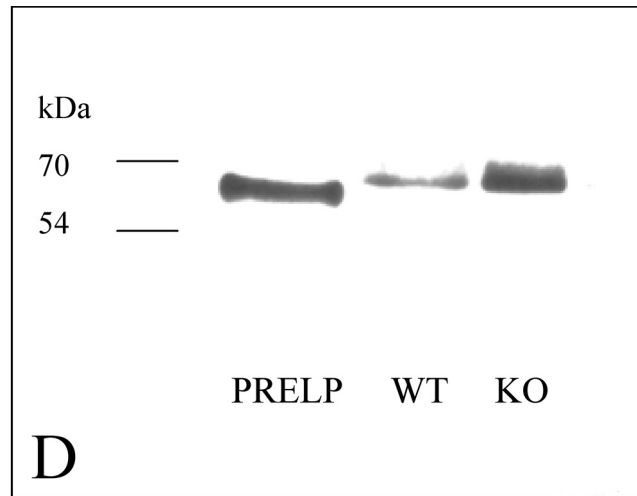
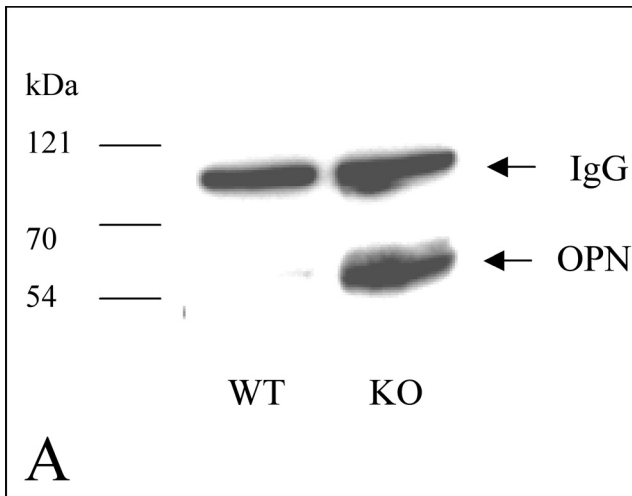


Fig. 6. Western blots demonstrating presence of OPN (A), COMP (B), decorin (C), PRELP (D), and fibromodulin (E) in aortic extracts from apo E/LDLr deficient (KO) and wild type mice (WT). Control lanes contain bovine COMP (B), mouse tracheal cartilage (C,E) and bovine PRELP (D).

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interaction with protein ligands (Kobe and Deisenhofer, 1994). Many of the ECM LRR proteins bind to collagen and appear to have roles in collagen fibrillogenesis. However, they may also regulate cellular functions such as adhesion, proliferation and migration, for reviews see (Iozzo, 1997; Hocking et al., 1998).

We found the LRR proteoglycan decorin in the lesions as well as in intact arterial wall, with the most abundant expression in the fibrous cap regions. This is in line with studies by Riessen et al., who has shown that decorin accumulates in atherosclerotic and restenotic lesions and mainly in association with collagen-rich regions of the plaque (Riessen et al., 1994). It has been shown that decorin binds to and inhibit collagen fibrillogenesis (Vogel et al., 1984). It may also retain LDL particles in the vessel wall (Kovanen et al., 1999) and recently, it was shown that decorin modulates matrix mineralisation (Mochida et al., 2003).

The LRR proteins PRELP and fibromodulin showed a similar distribution in the aorta. The expression was most prominent in lesions and particular in regions rich in macrophages. These LRR proteins are present in a number of different connective tissues, including cartilage and bone, but none of them has been studied in the vessel wall before. PRELP was not only present in

macrophage-rich lesions but also in SMC regions of plaques, indicating that it may have different functions than fibromodulin during the atherosclerotic process. PRELP and fibromodulin bind tightly to collagen and it is likely that they are necessary for an adequate collagen fiber formation, being a prerequisite for the development of a stable plaque. The LRR proteins, particularly PRELP, which contains a heparan sulphate binding sequence (Bengtsson and Aspberg, 2000), may bind to cells in the lesion and regulate their migratory and proliferatory behaviour.

In certain areas of advanced lesions we found distinct cartilage-like structures with expression of the cartilage specific proteins aggrecan and collagen II. Figures 2 and 7 demonstrates co-localization of collagen II and aggrecan with COMP, fibromodulin and PRELP. It is possible that the expression of COMP, fibromodulin, PRELP and other cartilage proteins precede the formation of cartilage structures in the vessel wall. A recent paper demonstrates that bone marrow derived stem cells induced to differentiate to chondrocytes express ECM proteins including COMP and fibromodulin (Barry et al., 2001). Previous reports have demonstrated presence of cells with chondrocyte-like appearance in arterial lesions of mice (Qiao et al., 1994)

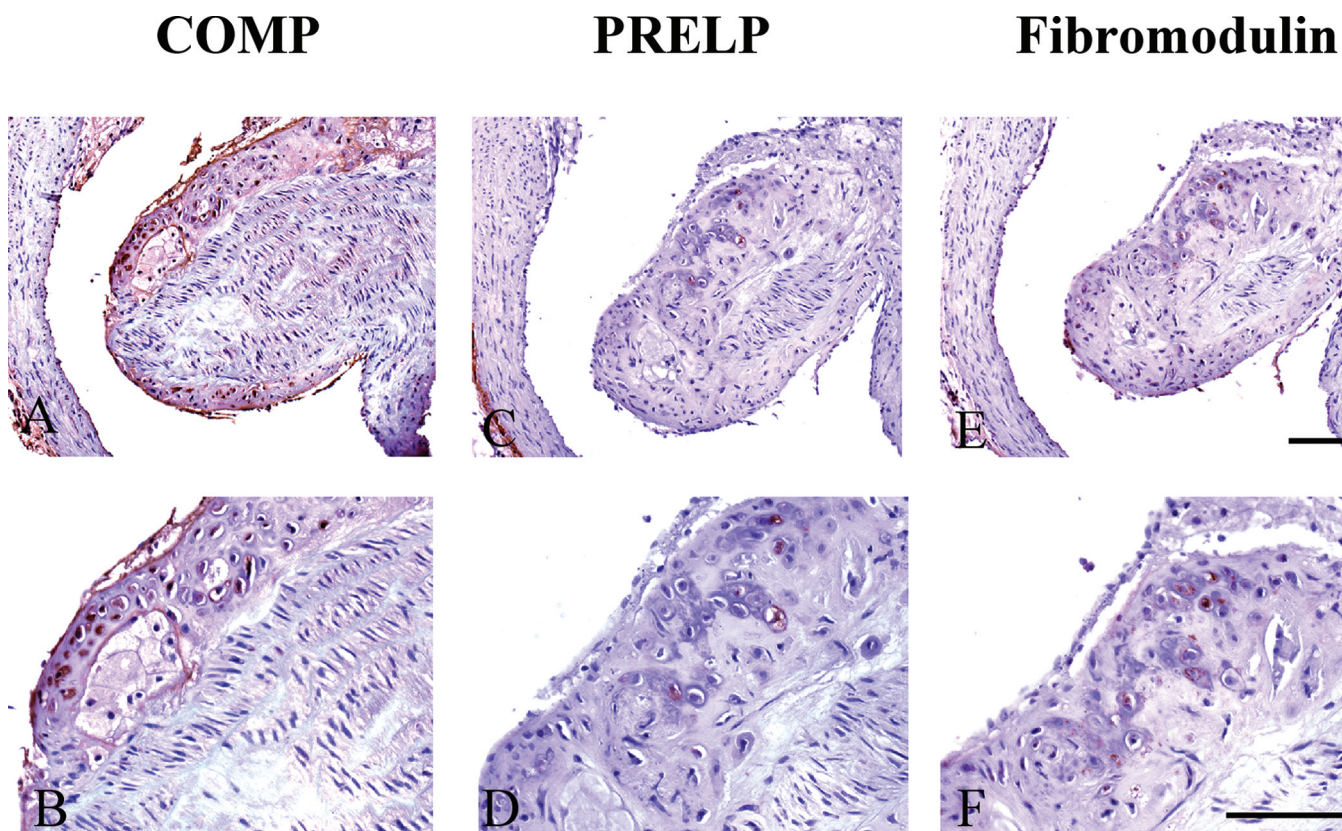


Fig. 7. Cartilage proteins in advanced lesion. Immunohistochemistry demonstrating presence of COMP (A and B), PRELP (C and D) and fibromodulin (E and F) in cartilage-like areas of an advanced lesion of a 24 weeks old apo E/LDLr deficient mouse. Scale bars: 100 μ m.

and humans (Qiao et al., 2003) in association with calcification. The chondrocyte-like cells may originate from pericytes of the adventitia (Canfield et al., 2000), neural crest-derived cells of the media (Topouzis and Majesky, 1996) or circulating stem cells from the bone marrow (Sata et al., 2002).

In summary, we have demonstrated the expression of a number of ECM proteins in atherosclerotic aortas of ApoE/LDLr deficient mice. OPN and COMP were found only in lesion areas, fibromodulin and PRELP were up-regulated in atherosclerotic aortas, whereas decorin was present in both normal and diseased vessels. All of these proteins are associated with cartilage and some of them co-localised with the cartilage-specific proteins collagen II and aggrecan in advanced lesions. The results suggest that cartilage-associated cell/collagen binding ECM proteins are involved in the pathogenesis of atherosclerosis, and may form a basis for future studies on functions of these ECM proteins in vascular disease.

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