Fibulin-2 is present in murine vascular lesions and is important for smooth muscle cell migration

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Abstract

Objective: The vascular extracellular matrix (ECM) can affect smooth muscle cell (SMC) adhesion, migration and proliferation—events that are important during the atherosclerotic process. Fibulin-2 is a member of the ECM protein family of fibulins and has been found to cross-link versican/hyaluronan complexes, an ECM network that has been suggested to be important during tissue repair. In this study we have analysed the presence of fibulin-2 in two different models of murine vascular lesions. We have also examined how the fibulin-2/versican network influences SMC migration.

Methods: Presence of fibulin-2 was analysed by immunohistochemistry in atherosclerotic aortas and in mechanically injured carotid arteries from mice. Fibulin-2 protein levels were also studied by Western blotting during rat aortic SMC phenotypic modulation in vitro. The importance of a fibulin-2/versican interaction for SMC migration was studied in the presence of two inhibiting peptides (FN III 3–5 and aggrecan C-type lectin-like domain).

Results: Fibulin-2 is expressed in SMC rich regions of atherosclerotic lesions where it colocalises with versican and hyaluronan. It is also present in injury-induced vascular lesions and is upregulated during SMC phenotypic modulation in cell culture. Moreover, treatments with peptides that block the interaction between versican and fibulin-2 inhibit SMC migration in vitro.

Conclusions: Fibulin-2 can be produced by SMC as a response to injury and may participate in the ECM organisation that regulates SMC migration during vessel wall repair.

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1. Introduction

The vascular extracellular matrix (ECM) not only provides mechanical strength to the normal vessel wall but is also involved in cellular activities that are important for the development of atherosclerosis and restenosis [1]. During these conditions, the ECM surrounding the smooth muscle cells (SMC) is altered which affects the adhesion, migration and proliferation of these cells, for review see [2].

Fibulin-2 is part of the fibulin-family [3], a group of ECM proteins in which several of the members are induced in response to injury, for review see [4]. It is a disulphide-linked dimer of 175 kDa subunits that can form two-, three-, and four-arm structures [5]. All of the fibulins, except fibulin-3, have been found present during cardiovascular development [6–8]. Furthermore, in a recent study, it was found that fibulin-5 deficient mice displayed enhanced SMC proliferation and migration [9]. Fibulin-2 is upregulated during epithelial-mesenchymal transformation in the developing heart and has been suggested to be involved during vascular remodeling [10]. Previous studies have shown that both fibulin-1 and fibulin-2 bind to versican and other
lectins via their C-type lectin like domain (CLD) [11,12]. Because of its multimeric structure, fibulin-2 and other CLD ligands have been suggested to cross-link the hyaluronan-proteoglycan complexes, which is thought to be important for ECM formation during development and in response to injury [12].

Versican is a proteoglycan that can be produced by synthetic SMC [13] and is upregulated in atherosclerotic and restenotic lesions [14,15]. It is composed of a central region with attached glycosaminoglycan (GAG) chains, an N-terminal globular domain and a carboxy-terminal globule which contains a CLD (for review, see [16]). Via its N-terminal domain, versican binds to hyaluronan (HA) [17] and form large pericellular coats that have been shown to be required for SMC migration and proliferation [18]. In view of the fact that versican has a significant importance during vascular disease, it is reasonable to believe that fibulin-2 may also be present in vascular lesions, and that they together play a role during atherogenesis.

We speculate that the network between versican and fibulin-2 can be important for SMC functions during vessel wall injury. In this study we have analysed the presence of fibulin-2 in murine vascular lesions and during phenotypic modulation of cultured rat aortic SMC. To clarify the significance of the fibulin-2/versican network for SMC function, we have also analysed SMC migration in presence of two peptides (FN III 3–5 and aggrecan C-type lectin-like domain) that inhibit the fibulin-2/versican interaction.

2. Materials and methods

2.1. 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 U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). Apo E−/−/LDLr−/− (B6.129–Apoem1UncLdlrtm1Her) and control mice (C57Bl6J) with the same background strain were purchased from JAX mice, Jackson Laboratories, Bar Harbour, ME. The animals were fed regular diet and given water ad libitum. ApoE/LDLr double knockout mice were sacrificed at the age of 8, 16 and 24 weeks by carbon dioxide inhalation. Periadventitial collar injury was performed on C57BL/6 mice.

2.2. Periadventitial collar injury

At the age of 21 weeks, mice were anaesthetised with Avertin (0.016 ml/g of 2.5% solution IP), and the right carotid artery was carefully isolated under a dissecting microscope. A nonocclusive plastic collar was placed around the right carotid artery, and the skin incision was closed, as described previously [19]. Mice were killed 21 days after collar placement and the carotid arteries were perfused with Histochoice (Amresco), dissected out and stored in Histochoice at 4 °C until analysis.

2.3. Antibodies and hyaluronan-detecting agent

A monoclonal anti mouse versican antibody, 12C5 [20] (Developmental Studies Hybridaom Bank), a monoclonal anti-mouse alpha-actin antibody (Sigma) and a polyclonal anti mouse fibulin-2 antibody [21] were used with appropriate secondary antibodies. Hylaronan was detected by a fluorescent fusion protein (neurocan-GFP) that specifically binds to hyaluronan [22].

2.4. Immunohistochemistry

Aortas from ApoE/LDLr double knockout mice and carotid arteries after periadventitial collar injury were dissected, dehydrated through increasing concentrations of ethanol followed by xylene, and embedded in paraffin (Histowax). Tissue sections (5 μm) were rehydrated with xylene and ethanol and pre-treated with hyaluronidase (2 mg/ml in PBS pH 5.0, 37, 15 min). When using primary antibody made in mouse, sections were first treated with Histomouse kit (Zymed Laboratories) according to manufacturers’ instructions, to prevent secondary antibodies to react with endogenous mouse IgG. The reaction products were visualised with the VECTASTAIN ABC Elite Kit (Vector Laboratories, Burlingham, CA, USA) using DAB as substrate (Vector Laboratories). Counterstaining was made with haematoxylin. The double immunofluorescent studies for fibulin-2 and versican were visualised with fluorescent secondary antibodies; Alexa Fluor 594 donkey anti rabbit IgG and Alexa Fluor 488 donkey anti mouse IgG (Molecular Probes, USA). Bisbenzimide was used to detect nuclei. Figures show pictures from representative experiments, which were repeated at least three times with similar results.

2.5. Cell culture

Rat SMC were isolated by 0.3% collagenase digestion [23], maintained in Hams F12 medium (Gibco) supplemented with 50 mg/l L-ascorbic acid, 50 mg/l gentamycin sulphate, 10% newborn calf serum (NCS, Gibco) and grown at 37 °C in a humidified atmosphere of 5% CO2 in air. Primary cultures represent cells that have not been passaged, while secondary cultures have been trypsinised once.

2.6. Protein isolation and Western blotting

For analysis of fibulin-2 expression during phenotypic modulation, SMC were harvested at 0, 2, 4 and 6 days in primary culture and in secondary culture. Cells at the time point 0 days were collected immediately after collagenase digestion. The cells were rinsed with PBS before they were transferred to extraction buffer (2% SDS in PBS). Fibulin-2 expression in aortas of ApoE/LDLr double knockout and
wild type mice was detected with Western blotting. Aortas were dissected clear (4–5 pooled aortas per experiment) ground in liquid nitrogen and extracted with 4 M guanidine hydrochloride buffer on a shaking table for 24 h. Samples were then centrifuged and the supernatant was precipitated twice in 10 volumes of 96% ethanol, 50 mM sodium acetate and dissolved in PBS. Smooth muscle cell lysates and homogenised aortas were separated on 10% SDS-polyacrylamide gels [24], with equal amount of total protein (30 μg per lane assessed according to the Bradford method). After electrophoresis, proteins were transferred onto Hybond-C nitrocellulose membrane (Amersham Lifescience) and equal loading was assured by staining the membrane with Ponceau S. The Ponceau S stained membranes were photographed before they were blocked overnight with 3% non-fat dry milk in PBS. The membranes were incubated 1 h at room temperature with antiserum against fibulin-2. After washing 3 × 10 min in PBS with 0.1% Tween (Sigma), the membranes were incubated with horseradish peroxidase-linked secondary antibodies. After subsequent washing, the bound antibody was detected using ECL plus Western blotting detection system (Amersham). Band density was determined using scanning densitometry (LAS 1000 Plus, Fujifilm).

2.7. RT PCR

Total RNA was extracted from SMC at 0, 2, 4 and 6 days in primary culture and in secondary culture according to the instructions of the RNeasy mini kit (Qiagen). RNA was transcribed to single-stranded cDNA by Superscript II (Invitrogen) using oligo dT primers. The cDNA was used as templates in PCR reactions with rat fibulin-2 primers (5′-TGT CAC GCG CAG ACT CAA CG-3′, 5′-CTT GGC CTG TAC TCA GTG CC-3′) and primers for the house keeping gene HPRT, hypoxanthine phosphoribosyltransferase (5′-CCT GCT GGA TTA CAT TAA AGC ACT G-3′, 3′-CTT GGC ATA TCC AAC AAG C-5′). The products were run on an agarose gel and detected by ethidium bromide.

2.8. Production of recombinant proteins

The construction of His-tagged aggre can CLD mammalian expression pCEP4 plasmid and production of the recombinant protein has previously been described [11,25]. The recombinant tenascin-R fragments constituted by FN III 1–2, FN III 3–5 and FN III 4–5 were produced as glutathione S-transferase (GST) fusion proteins [26]. These were expressed in Escherichia coli strain TG-1, isolated on glutathione-sepharose (Amersham Biosciences), released from its fusion partner by thrombin digestion and further purified on a mono-Q column as previously described [25].

2.9. SMC migration assay

Rat aortic SMC in secondary culture were grown to confluence on glass coverslips in six-well plates in F12 medium with 10% NCS supplemented with FN III 3–5, control fragment (FN III 1–2 or FN III 4–5) or aggre can CLD at indicated concentrations. After serum starvation (F12, 0.1% BSA together with FN III 3–5 or aggre can

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Fig. 1. Fibulin-2 expression in atherosclerotic aorta. Immunohistochemical detection of fibulin-2 in sectioned aorta from 24 weeks old wildtype mouse (A), 8 weeks old (B) as well as 24 weeks old (C and D) ApoE/LDL receptor deficient mice. Smooth muscle α-actin (E) and control staining (without primary antibody, F) were performed on adjacent sections. Asterisk indicates region of macrophages and arrows indicate regions of positive immunostaining. Scale bar=50 μm.
CLD for 24 h an in vitro injury was made by a pressure with a 3-mm wide soft plastic tube to the bottom of the culture dish. This injury, which produces a cell-free zone marked off by an elevated border of damaged as well as intact cells, induces migration and proliferation [27]. After 24 h, cells were fixed in 4% paraformaldehyde, stained with methylene blue and the number of migrating cells from one injury border was determined using light microscopy. The relative migration was determined by calculating the ratio between migrating cells with and without the addition of the individual fragments. Experiments were performed in triplicate and repeated at least twice.

2.10. Statistics

The statistical significance between groups was determined by Mann-Whitney test. P<0.05 was considered significant.

3. Results

3.1. Fibulin-2 is present in vascular lesions

By immunohistochemistry and Western blotting, we found presence of fibulin-2 in lesions of atherosclerotic aortas from 24 weeks old ApoE/LDL receptor knockout mice (Figs. 1 and 2). In aortas from wild type mice, staining for fibulin-2 was absent or weakly located to the internal elastic lamina (Fig. 1A). In 8 weeks old ApoE/LDL receptor...
knockout mice, where no lesions could be detected, fibulin-2 staining was pronounced in close association with the internal elastic lamina (Fig. 1B), whereas in 24 weeks old mice, fibulin-2 staining co-localised with positive smooth muscle α-actin staining (Fig. 1D and E). Fibulin-2 was notably absent in macrophage filled areas of the lesion (Fig. 1D). Western blotting confirmed increased levels of fibulin-2 in atherosclerotic aorta, a distinct band corresponding to the size of fibulin-2 was detected in extract of diseased aorta, while only a faint band appeared in wild type extract (Fig. 2).

In order to analyse the presence of fibulin-2 in mechanically-induced vascular lesions, we performed peri-adventitial collar injury in c57/Bl6 mice. Fibulin-2 was absent in uninjured arteries and in arteries 3 days after injury, whereas it was present in neointimal lesions in arteries 9 and 21 days after injury. In these arteries, fibulin-2 was also present in the media (Fig. 3).

3.2. Fibulin-2 is produced by SMC and is upregulated during SMC phenotypic modulation

In cell culture, SMC undergo phenotypic transition from a contractile to a synthetic state [28]. In order to study fibulin-2 expression during SMC phenotypic modulation, we analysed SMC after different time points in culture. By Western analysis, we found that fibulin-2 expression was absent after 0 and 2 days in primary culture, whereas it was detected in increasing amounts in cells from 3, 4 and 6 days in primary culture as well as in secondary culture (Fig. 4A and B). In RT PCR experiments, fibulin-2 expression was found at all time points, although with the lowest expression in freshly isolated cells and in increasing levels during the phenotypic modulation, at days 2, 4 and 6 (Fig. 4C).

3.3. Fibulin-2 colocalises with versican and hyaluronan in atherosclerotic lesions

By immunohistochemistry and with the use of a GFP-coupled hyaluronan-binding peptide, we found that fibulin-2 colocalised with versican and hyaluronan in atherosclerotic lesions. Fibulin-2, versican and hyaluronan expressions were all located in the outermost luminal part of lesions (Fig. 5). There was no positive staining in the media of atherosclerotic (Fig. 5) or wild type aorta (data not shown).

Fibulin-2 and hyaluronan levels were also detected in migrating SMC after mechanical injury in vitro. Fibulin-2 was found pericellularly in a distinct fibrous-like pattern around the cells that were migrating out in the wounded area (Fig. 6A). The staining for hyaluronan was also found pericellularly, but with a more widespread distribution and with some hyaluronan staining found in association with the cells (Fig. 6B). Fibulin-2 and hyaluronan staining colocalised in some regions although primarily, the two components were located adjacent to each other (Fig. 6D).

3.4. SMC migration is reduced in presence of peptides that inhibit the fibulin-2/versican interaction

The finding that fibulin-2 colocalised with versican in atherosclerotic lesions rose the question whether this network may be important for SMC functions. In order to analyse the role of the fibulin-2/versican interaction for SMC migration we used two recombinant peptides (FN III 3–5 and aggrecan CLD) in an in vitro SMC migration assay. FN III 3–5 is a fragment of tenascin-R that contains the binding site to versican CLD, acting as a competitive inhibitor to fibulin-2/CLD binding [12]. The aggrecan CLD peptide on the other hand, competes with versican for binding to fibulin-2. SMC migration was measured as the number of cells migrating out from the border of a wound formed after a pressure-induced in vitro injury.

It was found that injury-induced migration was inhibited by the presence of 3 μM FN III 3–5 (Fig. 7A). The control fragment (FN III 1–2 or FN III 4–5), which do not bind proteoglycan CLD [25], had no effect on cell migration (Fig. 7A). To confirm that the observed effect of FN III 3–5 was due to inhibition of versican/CLD interactions, we...
investigated the effect of aggrecan CLD on SMC migration. The migratory capacity of SMC was inhibited by the presence of 300 nM aggrecan CLD (Fig. 7B). Migrating cells were not only reduced in number, but also exhibited an altered morphology. This is demonstrated by a more elongated spindle-like morphology of cells cultured in
presence of FN III 3–5 compared to control cells (Fig. 8). Similar to FN III 3–5, presence of aggrecan CLD also affected SMC morphology, resulting in migrating cells with an elongated spindle-like morphology (Fig. 8). In order to ensure that the inhibiting fragments (FN III 3–5 and CLD) did not affect cell viability/proliferation, an MTT assay [29] was performed, which showed no effect of the peptides on viable cell number (data not shown).

4. Discussion

In this study we demonstrate that fibulin-2 is present in murine vascular lesions and that fibulin-2 is upregulated during phenotypic modulation of SMC. Within the atherosclerotic lesions, it colocalises with versican and hyaluronan. Furthermore, we show that blocking the interaction between versican and fibulin-2 leads to decreased migration of SMC.

We found increased levels of fibulin-2 in atherosclerotic aorta from apoE/LDLr deficient mice. To our knowledge, this is the first report demonstrating presence of fibulin-2 in atherosclerosis. The expression of fibulin-2 was particularly prominent in the fibrous cap region of lesion and it was notably absent in regions with macrophages. In normal aorta, fibulin-2 expression was restricted to the internal elastic lamina, which confirms previously published findings by Reinhardt et al. [30].

Fibulin-2 was also found to be produced by rat aortic SMC during phenotypic modulation in cell culture. In freshly isolated cells and in cells from day 2 in primary culture, fibulin-2 was absent, while it was present in cells from days 4, 6 and in secondary cultures. This result is in line with the finding that fibulin-2 is present in higher amounts in lesions than in the media, as lesion-derived SMC are in a synthetic state whereas SMC in normal media have a contractile phenotype. Fibulin-2 has previously been found to be a specific marker for activated myofibroblasts [31] and it is present during epithelial–mesenchymal transformation during heart development [10]. Fibulin-2 may thus play a role during cell transitions at sites of injury.
and during development. The finding that fibulin-2 is upregulated during SMC phenotypic modulation together with the result that it is present in SMC rich regions in lesions suggest that arterial SMC synthesise fibulin-2 as a response to injury.

We speculate that fibulin-2 is produced by synthetic SMC in order to link extracellular networks of versican and hyaluronan and that these complexes are important for SMC function. This hypothesis is supported by the fact that fibulin-2 is a dimer and has been shown to cross-link lectican–hyaluronan complexes [12]. In addition, in a study by Tsuda et al., it has been suggested that fibulin-2 fibrillar networks promote migration, proliferation and differentiation of mesenchymal cells during development [10]. Fibulin-2 has also been showed to interact with other proteoglycans present in atherosclerotic lesions, such as perlecan [32]. However, this interaction have not been studied in the vascular wall. Furthermore versican and perlecan bind to different regions of fibulin-2 [12,32], thus it is unlikely that the inhibiting peptides used in the present study would effect a potential fibulin-2/perlecan interaction.

The fibulins are often associated with basement membranes and elastic fibers in different tissues, including the cardiovascular system. Since fibulin-2 binds to elastin and fibillin-1 it may function in anchoring microfibrils to elastic fibres, for review see [4]. Our results show that fibulin-2 is absent in normal arterial wall and in freshly isolated SMC, which are conditions in which basement membrane associated proteins are expected to be present [33]. The present results suggest that fibulin-2 may play an additional role during ECM organisation and in cell-matrix interactions beside its role in basement membranes and during elastic fiber assembly.

Our data demonstrate that fibulin-2 colocalise with versican and hyaluronan in atherosclerotic lesions. Versican has previously been found to colocalise with fibulin-2 in developing hearts and both have been suggested to be involved in ECM organisation during development [6]. Furthermore, versican has been detected in human atherosclerotic plaques [14]. Conversely, in a study by Kunjathoor et al., versican was demonstrated absent in lesions from ApoE deficient mice and LDLr deficient mice [34]. The lack of versican was suggested to be due to the macrophage-rich nature of lesions in these mouse models. However, in a previous study performed in our laboratory, we show presence of both macrophages and SMC in lesions of the ApoE/LDLr double deficient mouse [35].

The presence of hyaluronan in atherosclerotic lesions has been reported previously (for review see [36]) and overexpressing hyaluronan in the tunica media has been shown to promote the development of atherosclerosis in ApoE−/− mice [37]. Hyaluronan can be produced by SMC [38] and binds to cells through CD44 and receptor for hyaluronan mediated motility (RHAMM) [39], both present on SMC. It has also been shown to affect SMC migration in vitro [40].

By using an in vitro injury assay, we found that SMC migration was reduced in the presence of FN III 3–5. Since FN III 3–5 is a competitive inhibitor for the binding between versican and fibulin-2 [12], it is possible that the complex versican–hyaluronan/fibulin-2 is essential for appropriate migration. Indeed, in a recent paper by Olin et al., it was suggested that one of the functions of the CLD is to organise the forming hyaluronan–lectican complexes in the assembly of the ECM [12]. Furthermore, it has been shown that the versican–hyaluronan complex is essential for SMC function and blocking the binding of hyaluronan to SMC inhibits both proliferation and migration [18,40]. Fibulin-2 may thus play an important role for the organisation of this ECM network and influence the behaviour of the cell. The fact that the addition of CLD also inhibited SMC migration supports our hypothesis concerning the importance of the versican/fibulin-2 interaction for SMC function.

As there exist several ligands to versican CLD, the presence of FN III 3–5 may affect other CLD interactions. Tenascin-C is another CLD-ligand [41] that can be synthesised by SMC [42], however the significance of the tenascin-C/versican interaction for SMC function has not yet been studied.

In summary we have demonstrated that fibulin-2 is present in vascular lesions and colocalise with versican and hyaluronan in atherosclerotic plaques. Furthermore, we have showed that the interaction between fibulin-2 and versican may be important for SMC migration. We propose that fibulin-2 is produced by SMC in response to injury and that the versican/fibulin-2 network is important for SMC function during vessel wall repair.

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