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Retrovirally Mediated Overexpression of Glycosaminoglycan-Deficient Biglycan in Arterial Smooth Muscle Cells Induces Tropoelastin Synthesis and Elastic Fiber Formation *in Vitro* and in Neointimae after Vascular Injury

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Galactosamine-containing glycosaminoglycans (GAGs), such as the chondroitin sulfate chains of the proteoglycan versican, have been shown to inhibit elastogenesis. Another proteoglycan that may influence elastogenesis is biglycan, which possesses two GAG chains. To assess the importance of these chains on elastogenesis in blood vessels, rat aortic smooth muscle cells were transduced with a GAG-deficient biglycan cDNA-containing retroviral vector (LmBSN). Control cells were transduced with either biglycan or empty vector. Transduced cells were characterized *in vitro* and then seeded into balloon-injured rat carotid arteries to determine the effects on neointimal structure. Cultured cells overexpressing LmBSN showed marked up-regulation of tropoelastin and fibulin-5 mRNAs, increased amounts of desmosine and insoluble elastin, and increased deposition of elastic fibers as compared with empty vector- and biglycan-transduced cells. Conversely, collagen $\alpha(1)$ synthesis and the deposition of collagen fibers were both markedly de-

creased in LmBSN cultures. *In vivo*, neointimae formed from cells that overexpressed LmBSN and showed increased deposits of elastin that aggregated into parallel nascent fibers, generally arranged circumferentially. Neointimae that formed from cells with biglycan or empty vector contained fewer and less aggregated deposits of elastin. These findings suggest that the GAG chains of biglycan serve as inhibitors of elastin synthesis and assembly, and that biglycan can act as an important modulator of the composition of the extracellular matrix of blood vessels. (Am J Pathol 2008; 173:1919–1928; DOI: 10.2353/ajpath.2008.070875)

Several recent studies have highlighted the reciprocal relationship between elastogenesis and matrix proteoglycan content of tissues.^{1–5} Elastic fibers are generally absent or depleted in matrices rich in chondroitin sulfate (CS)-containing proteoglycans and correspondingly increased in matrices depleted of CS proteoglycans. Versican, with its multiple CS glycosaminoglycan (GAG) chains, has been shown to be an effective inhibitor of elastogenesis.⁵ Overexpressing the versican variant that lacks CS chains in aortic smooth muscle cells and in skin fibroblasts promotes tropoelastin synthesis and elastin assembly,^{3,4} consistent with a role for CS in inhibiting the assembly of elastic fibers. Previous studies have suggested that CS chains interact with the tropoelastin chaperone elastin binding protein to decrease the delivery of tropoelastin to growing fibers.^{1,5}

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Another proteoglycan that could potentially effect elastogenesis and decrease elastic fiber assembly is biglycan. Biglycan possesses two GAG chains containing chondroitin and dermatan sulfates. For example, dermatan sulfate has been shown to decrease elastogenesis in Hurler disease.² Several studies, however, have proposed that biglycan may enhance rather than decrease elastogenesis. Biglycan core protein binds to tropoelastin and to elastic fiber microfibrils,⁶ and in injured kidney, biglycan stimulates fibrillin-1, a major component of the microfibrils that form the scaffold on which tropoelastin is deposited.⁷ In abdominal aortic aneurysms, where elastin is disrupted and fragmented, biglycan gene expression is decreased.⁸ In addition, recent studies show that biglycan deficiency coincides with spontaneous aortic dissection and rupture in mice,⁹ indicating a key role for this proteoglycan in vascular wall structure. On the other hand, biglycan stimulates proliferation and migration,¹⁰ and induces cell elongation, features associated with a non-elastogenic phenotype.¹¹

To explore further the role of biglycan and the importance of the GAG chains in elastogenesis, we have compared the elastogenic potential of aortic smooth muscle cells overexpressing normal biglycan with counterparts overexpressing mutated biglycan in which the two GAG attachment sites on the core protein were mutated to preclude chain attachment. Using the retroviral vector LXS_N, we transduced cultured rat smooth muscle cells with the GAG-deficient human biglycan (LmBS_N), as well as normal human biglycan (LBS_N) and the empty vector (LXS_N) as an additional control. Following characterization of the retrovirally modified cells in culture, cells were seeded into balloon-damaged carotid arteries of adult rats to investigate effects on elastogenesis in neointima formation. We report that overexpression of GAG-deficient biglycan promotes elastogenesis *in vitro* and *in vivo*. Furthermore the increased elastogenesis is accompanied by decreased collagen synthesis and collagen fiber deposition, thus altering the balance of components in the extracellular matrix in vascular tissue.

Materials and Methods

Retroviral Vectors and Construction of Mutant Biglycan

The cDNA of human biglycan (courtesy of Dr. Marian Young, Craniofacial and Skeletal Disease Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, MD)^{12,13} was inserted into the EcoR1 site of the replication defective retroviral vector LXS_N (courtesy of Dr. A. D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA)¹⁴ to create the biglycan-expressing vector (Figure 1).

To assess the importance of the biglycan GAG chains to aortic smooth muscle cell phenotype and elastin metabolism, site-directed mutagenesis was used to create mutant biglycan cDNA in which the serines at the two GAG attachment sites of the biglycan core protein were replaced by alanine residues.¹⁵ Two complimentary oli-

Figure 1. Schematic depiction of retroviral vectors LXS_N, LBS_N, and LmBS_N. LTR, long terminal repeat; NEO, neomycin phosphotransferase; SV, SV40 fragment containing early promoter; pA, polyadenylation site; hbiglycan, human biglycan cDNA; mhbiglycan, mutant human biglycan cDNA. **Arrows** indicate transcriptional start sites and direction of transcription. **Arrowheads** indicate sites of serine to alanine mutations, to prevent glycosaminoglycan side chain binding.

gonucleotides were used for the mutagenesis reaction: forward 5'-GAACGATGAGGAAGCTGCGGGCGCTGAC-ACCGCAGGCGTCCTGGACC-3' reverse 5'-GGTCCAG-GACGCCTGCGGTGTCAGCGCCCGCAGCTTCCTCAT-CGTTC-3'.

These primers encode for T to G substitutions at the bases underlined on the forward primer, thus encoding for alanines rather than serines at the fifth and tenth residues of the biglycan core protein. The two mutagenic primers were used in a PCR using pBluescript plasmid containing the native human biglycan cDNA as a template. The products of the PCR were treated with the methylation-sensitive reaction enzyme DpnI to digest the parental DNA plasmid template.

Enriched mutant plasmids were transfected to *E. coli*, and the resultant colonies screened for mutated cDNA clones. The full-length mutated biglycan cDNA was sequenced to confirm the presence of the intended mutated biglycan sequence, and the *E. coli* fragment of mutated biglycan was inserted into the LXS_N vector.

LXS_N vectors containing human biglycan cDNA, or mutated human biglycan, were transfected into 317 packaging cells and resultant viruses LBS_N and LmBS_N as well as empty vector (LXS_N) used to transduce Fisher 344 rat aortic smooth muscle cells as described previously.^{14,16,17}

Cell Culture

Aortic smooth muscle cells were obtained and cultured as previously described.^{16,17} Transduced cells were maintained in Dulbecco's minimal essential high glucose medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, glutamine and penicillin-streptomycin (Invitrogen, Carlsbad, CA). Cells were used between 4 and 8 passages after the initial transduction.

Northern Blot Analysis

For Northern analysis, 7.5×10^5 cells were plated in 60 mm dishes and cultured for 14 days. Total RNA was extracted from cells by RNAeasy Mini Kits (Qiagen, Valencia, CA). Twelve micrograms per sample of total RNA was run on a 0.8% agarose gel containing formaldehyde, then subjected to limited hydrolysis, transferred to Zeta-probe (Bio-Rad), and cross-linked by UV light. Membranes were probed as described previously.¹⁸ Full-length human biglycan¹² was used to detect all endogenous rat biglycan, human biglycan, and mutated human biglycan mRNA. Tropoelastin mRNA was detected with a probe generously provided by Dr. C. D. Boyd, University of Hawaii, Manoa, Honolulu, Hawaii.¹⁹ Collagen $\alpha(1)$ was detected with human pro $\alpha(1)$.²⁰

Quantitative Real-Time Reverse Transcriptase PCR

DNA free-total RNA was obtained from cultured cells using the Total RNA Isolation Kit (Agilent Technologies, Santa Clara, CA) as directed by manufacturer. cDNA was prepared from 1 microgram total RNA and reverse transcribed in a 40 μ l reaction mix with random primers using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to manufacturer instructions. Carotid cDNA was obtained as described in quantitative real-time reverse transcriptase PCR (QRT-PCR) Detection of seeded cells methods section. Relative quantitation of gene expression was performed using TaqMan Gene Expression Assays (Applied Biosystems) for tropoelastin Rn01499783_m1, collagen $\alpha(I)$ Rn01504536_m1, fibulin-5 Mm01336252_m1, and fibrillin-1 Rn00582774_m1. Gene expression was normalized to eukaryotic 18S rRNA endogenous control (part #4333760, Applied Biosystems). Briefly, 20 ng cDNA was amplified in 1 $\times$ TaqMan Fast Universal PCR Mix with 250 nmol/L TaqMan probe in a 20 μ l reaction using the Fast program for 50 cycles on an ABI7900HT machine. All samples were in duplicate and data were analyzed using the Comparative Ct Method using software from Applied Biosystems. Data from cell culture were averaged from two different experiments of duplicate dishes of cells from two separate thaws and passage numbers done at separate times. QRT-PCR of tropoelastin, collagen $\alpha(1)$, fibulin-5, and fibrillin-1 in the cultured cells was performed at 2, 3, 7, and 14 days with similar results.

Western Analysis

For Western analysis of biglycan core protein, 48-hour conditioned media from 14-day cultures, seeded at 7.5×10^5 in 60-mm dishes, were collected. To confirm the absence of GAG chains on biglycan secreted by LmBSN cells, samples of conditioned medium were passed over 0.5 ml DEAE-Sephacel columns in 8 mol/L urea, 0.5% Triton X-100, 0.01 Tris-HCL, pH 7.5, and 0.25% mol NaCl (urea buffer), washed with 10 volumes of urea buffer, and

eluted with 3 mol NaCl in urea buffer to collect any biglycan with GAG chains attached.

Western blot analysis was performed as described previously.^{21,22} Briefly, following the addition of 30 mg carrier chondroitin sulfate, eluted material was precipitated at 20°C by addition of 3.5 volumes of 95% ethanol containing 1.3% potassium acetate. The pellet was dissolved in distilled water and the ethanol precipitation was repeated without the addition of carrier. Following centrifugation the supernatant was discarded and the pellet was air-dried. Samples were resuspended in 8 mol/L urea. Chondroitinase digestion with ABC lyase (0.02 U) was performed in Tris buffer at pH 8 for 3 hours at 37°C. Samples were boiled for 5 minutes in SDS-containing sample buffer with β -mercaptoethanol.

All samples were run on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (BA83, Schleicher and Schuell Bioscience, Inc., Keene, NH) and exposed to primary antibody against human biglycan (LF51, a kind gift from Dr. Larry Fischer, Craniofacial and Skeletal Disease Branch National Institute of Dental Research, National Institutes of Health, Bethesda, MD). Following incubation with an alkaline phosphatase-conjugated secondary antibody, biglycan core protein was detected by enzyme-linked chemiluminescence (Tropix Inc. Applied Biosystem Bedford, MA).

Desmosine Analysis

The desmosine content of LXSBN, LmBSN, and LBSN cells cultured for 14 days, in triplicate, was determined using a radioimmunoassay as described previously.²³

Insoluble Elastin

Insoluble elastin in the cell layers of cultures was measured as previously described.² Briefly, LXSBN, LBSN and LmBSN cells were grown to confluency in 100 mm dishes in quadruplicates and [³H]-valine (20 μ Ci) added to each dish with fresh media. Cultures were incubated for 72 hours before removing the media and scrapping the cell layers in 0.1N NaOH, sedimented by centrifugation, and boiled in 0.5 ml of 0.1N NaOH for 45 minutes to solubilize all matrix components except elastin. Pellets of elastin were solubilized by boiling in 200 μ l of 5.7N HCL for 1 hour, and aliquots mixed with scintillation fluid and counted. Aliquots from each culture were also taken for DNA determination using DNeasy Tissue System from Qiagen. Results were normalized to DNA content and expressed as cpm/ μ g DNA.

Immunocytochemistry

Polyclonal rabbit antisera to the human $\alpha(1)$ c-telopeptide of collagen I was purchased from Chemicon (Temecula, CA). Polyclonal rabbit antisera to recombinant bovine tropoelastin, which also recognizes rat elastin, was purchased from Elastin Products (Owensville, MI; Cat No PR 396). Immunocytochemistry of cultured cells was performed as described previously.⁴

Balloon Injury and Cell Seeding

Carotid artery balloon injury and cell seeding were performed in Fisher 344 rats as described previously.^{16,17,24} All surgical procedures were performed according to the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, publication No. 86-23, revised 1985). Ten animals were used for each group (LXSN, LBSN, LmBSN) at each time point (2 and 4 weeks). At week 2, 6 animals from each group were used for light microscopy analysis, 2 animals for electron microscopy, and 2 for QRT-PCR. At week 4, 8 animals were used for light microscopy analysis and 2 animals for electron microscopy.

QRT-PCR Detection of Seeded Cells

RNA was extracted from frozen and homogenized pooled carotid arteries by the method of Chomczynski and Sacchi.²⁵ One microgram of total RNA was reverse transcribed and amplified using random hexamer primers and the Superscript Preamplification System Kit (Gibco/BRL Div. of Invitrogen, Gaithersburg, MD). To detect LXSN cDNA, a 510 base DNA fragment of LXSN was amplified (LXSN forward primer 1573, 5'-CCTTGAACCTCCTCGTTCGAC-3' 1593; LXSN reverse primer 2079, 5'-TCTTGTTCAATCATGC-GAAACG-3' 2061). To detect LBSN and LmBSN cDNA, a 986-base DNA fragment of LBSN and LmBSN cDNA were amplified (LXSN forward primer 1573, 5'-CCT TGA ACC TCC TCG TTC GAC-3' 1953; human biglycan reverse primer, 5'-GTA CAG CTT GGA GTA GCG AAG CA-3', 866). 35 PCR cycles were performed.

Histochemistry, Immunohistochemistry, and Electron Microscopy

Vessels for immunohistochemistry were perfusion fixed with 10% neutral-buffered formalin at 120 mm Hg pressure and sections from the paraffin-embedded carotids stained with H&E, Masson's Trichrome, and orcein to show general morphology, collagen and elastin respectively.³ Human biglycan and GAG-deficient human biglycan were detected with polyclonal antisera against recombinant human biglycan, LF121 (kind gift from Dr. Larry Fischer). Vessel segments for electron microscopy were fixed in 3% glutaraldehyde in 0.1 mol/L cacodylate buffer, secondarily fixed in 1% OsO₄, processed, and sectioned at right angles to the vessel axis.²⁶ Thin sections were mounted on formvar-coated grids, stained with uranyl acetate and lead citrate and viewed on a JOEL 1200 EXII microscope.

Morphometry

Immunostained fluorescent elastin and collagen in cell cultures was quantified using Image-Pro Plus software from Media Cybernetics (Silver Springs, MD). Quadruplicate cultures, from three separate experiments, were examined with a Nikon Eclipse E1000 microscope attached to a cooled CCD camera (QImaging, Retiga EX).

For each experiment 100 fields were analyzed under a 63× immersion objective. Volume fractions for elastin in the neointimae of seeded vessels were determined by point counting (100 point grid) of electron micrographs (magnification 10K) collected from two animals in each group, 4 weeks postseeding. A total of 30 random micrographs, taken in the mid/basal region of the neointimae, were analyzed (3000 points).

Data were analyzed by Student's *t*-test and by analysis of variance. A value of *P* < 0.05 was taken as significant.

Results

Expression and Secretion of Human Biglycan and GAG-Deficient Human Biglycan

mRNA for endogenous rat biglycan and mRNAs for retrovirally introduced human biglycan and GAG-deficient biglycan were expressed and distinguishable by Northern blot in early (4-day) and extended (14-day, Figure 2A) cultures of Fisher rat smooth muscle cells. Human biglycan core protein, isolated from 48-hour conditioned media from 14-day LBSN cultures, and digested with chondroitin ABC lyase, was detected on Western blot using antibody LF 51 that recognizes core protein of human but not rat biglycan (Fig-

Figure 2. A: Expression, at day 14, of rat biglycan (LXSN), human biglycan (LBSN), and mutated human biglycan (LmBSN). Northern blot probed with cDNA of human biglycan (hbi) that recognizes endogenous rat biglycan (rbi), and the mutated human biglycan. Endogenous rat and human biglycan mRNA is 1.7kb, increased to 2.5kb following transduction with the LXSN vector due to inclusion of the neomycin phosphotransferase sequence (794bp). B: Western blot of biglycan core protein isolated from media conditioned for 48 hours before collection at day 14, digested with chondroitin ABC lyase, and detected by antibody LF51 that recognizes the core protein of human but not rat biglycan. C: Western blot of samples collected over DEAE column before chondroitinase digestion, showing lack of binding of mutated human biglycan due to lack of GAG chains. D: SDS gradient polyacrylamide gel (4% to 12%) loaded with equal counts (25×10^3 dpm) of [³⁵S] sulfate-labeled secreted proteoglycans, showing overexpression of biglycan (bi) by LBSN cells compared with LXSN cells and reduction of sulfate-labeled chains by LmBSN cells.

Figure 3. **A:** Expression of tropoelastin mRNA. Northern blot of 14-day cultures probed with tropoelastin cDNA showing increased expression of message in LmBSN cells compared with LXSN and LBSN cells. **B:** Fold difference in tropoelastin mRNA, determined by QRT-PCR, showing significantly increased message in 14 day LmBSN cultures compared with LXSN and LBSN cultures. **C:** Quantitative analysis of elastin-specific desmosine content in x-day cultures showing a significant increase in LmBSN compared with LXSN and LBSN cultures. **D:** Quantitative analysis of immunoprecipitable [³H]-valine labeled insoluble elastin showing significant increase in 7 day LmBSN cultures and significant decrease in LBSN cultures. Error bars in this Figure and subsequent figures are SEM.

Figure 4. **A:** Fold difference in fibulin-5 mRNA, determined by QRT-PCR, showing significantly increased message in 14-day LmBSN cultures compared with LXSN and LBSN cultures. **B:** Fold difference in fibrillin-1 mRNA, determined by QRT-PCR, in 14-day cultures showing similar levels for LXSN, LBSN and LmBSN cultures. **C:** Expression of collagen I α(1) mRNA. Northern blot of 14-day cultures probed for collagen I α(1) showing decreased expression by LmBSN cells. **D:** Fold difference in collagen I α(1) mRNA, determined by QRT-PCR, showing significant decrease in message in 14-day LmBSN cultures compared with LXSN and LBSN cultures.

Figure 5. A: Immunostaining for elastin and Type I collagen in 10-day cultures of LXSN, LBSN, and LmBSN, showing decreased elastin and increased Type I collagen by cells overexpressing human biglycan, compared with both LXSN and LmBSN cells. Increased deposition of elastin, organized as fibers, and decreased collagen by LmBSN cells were observed compared with LXSN and LBSN cells. **B:** Quantitative morphometric analysis (see Methods) of elastin and collagen immunostaining, determined from quadruplicate cultures of LXSN, LBSN, and LmBSN cells in three separate experiments, confirming the changes shown in images in **A**.

ure 2B). Passage of media samples over a DEAE column before chondroitin ABC lyase digestion showed that GAG-deficient human biglycan was not retained on the column, confirming the absence of GAG chains (Figure 2C). Polyacrylamide gel electrophoresis of [35 S] sulfate-labeled secreted proteoglycans further confirmed the absence of GAG chains on biglycan produced by the LmBSN cells as well as confirmed the increase in total biglycan in the LBSN overexpressing cells (Figure 2D).

Tropoelastin Expression, Desmosine Content, and Insoluble Elastin Production

Both Northern blot of mRNA isolated from 14-day cultures and hybridized with a human tropoelastin probe that also recognizes rat tropoelastin and QRT-PCR of tropoelastin mRNA showed a significant increase in mRNA in the LmBSN cells as compared to LXSN-transduced cells (Figure 3, A and B). On the other hand, by Northern blot, the tropoelastin mRNA level in the LBSN cells was de-

creased by approximately 25% compared to LXSN-transduced cells. Similar results were obtained for 4-day cultures (data not shown). Cross-linked and insoluble elastin, measured by desmosine content (Figure 3C) and [3 H]-valine incorporation (Figure 3D) respectively, were also significantly higher in the LmBSN cultures compared with LXSN control and LBSN cultures. Insoluble elastin was significantly reduced in the LBSN cultures compared with control and LmBSN cultures (Figure 3D).

Expression of Elastin-Associated Proteins and Collagen $\alpha(I)$

Message levels for fibulin-5 and fibrillin-1 were determined by QRT-PCR and expressed as fold difference from LXSN control cells. Fibulin-5 mRNA (Figure 4A) was significantly increased compared with LXSN and LBSN cells whereas the levels for fibrillin-1 mRNA (Figure 4B) were similar across all groups. In contrast to tropoelastin message, collagen $\alpha(I)$ mRNA in the human GAG-defi-

Figure 6. A: Human anti-biglycan immunostaining of 2-week- and 4-week-old neointimae formed from transduced cells seeded into balloon injured rat carotid artery. Human biglycan (LBSN) and mutated biglycan (LmBSN) are present through the neointimae. Neointimae formed from vector alone cells (LXSN) are negative. **Arrowheads** indicate internal elastic lamina. Magnification = original $\times 40$. **B:** Expression of transgenic human biglycan *in vivo*; reverse transcription-PCR for LXSN vector and human biglycan mRNA at 4 weeks after seeding. Lane 1, conditional negative control; lane 2, LXSN cell-seeded vessel with LXSN and LXSN primers; lane 3, LBSN cell-seeded vessel and lane with LXSN and biglycan primers; 4, LmBSN cell-seeded vessel with LXSN and biglycan primers. **C:** Fold difference in tropoelastin mRNA, determined by QRT-PCR of 4-week-old vessel wall from LXSN, LBSN, and LmBSN seeded carotid arteries showing increased message in LmBSN carotid.

cient biglycan producing cells, determined both by Northern analysis and QRT-PCR, was markedly and significantly reduced compared with both LXSN and LBSN cells (Figure 4, C and D, respectively).

Immunostaining of Elastin and Collagen Type I

Differences in elastin immunostaining of cultured cells reflected the proportional differences in the mRNA expression patterns and the insoluble elastin levels. Elastic fibers were present in both LXSN and LmBSN cultures, but the latter displayed both more and better defined fibers (Figure 5A). In contrast, elastin staining was markedly reduced in the LBSN cultures. Immunostaining also confirmed the marked reduction in Type I collagen production in the LmBSN cultures compared with both the LXSN and LBSN cultures (Figure 5A). Notably, the LBSN cultures showed increased collagen deposition. Quantification of these immunostaining patterns by image analysis confirmed these differences (Figure 5B).

Expression and Production of Human Biglycan and Tropoelastin mRNA in Balloon-Injured Seeded Vessels

Immunostaining of 2- and 4-week-old seeded carotids with LF121, a polyclonal antibody that recognizes human biglycan, demonstrated strong staining at both time points in LBSN and LmBSN neointimae, and negative staining in the medial and adventitial layers as well as in the LXSN neointimae (Figure 6A). Staining was generally evenly intense through the width of neointimae, but with

slightly stronger staining in the mid to deep intima. QRT-PCR of carotid vessels 4 weeks after injury and cell seeding demonstrated the presence of appropriately sized transcripts for LXSN, LBSN, and LmBSN (Figure 6B). Analysis of tropoelastin mRNA by QRT-PCR of 4 week neointimae showed a fivefold increase for tropoelastin message compared with LXSN and LBSM vessels (Figure 6C).

Accumulation of Elastin in Balloon-Injured Seeded Vessels

Orcein staining of 2-week seeded carotids demonstrated elastin deposits in the neointimae of all vessels (Figure 7). The LXSN and LBSN seeded vessels contained numerous punctuate and generally small deposits of elastin scattered throughout the extracellular matrix of the neointimae. In contrast, LmBSN neointimae contained more elastin and larger deposits, often aggregated into bands or nascent fibers arranged parallel to the internal elastic lamina.

The increased deposits of elastin in the LmBSN neointimae, compared with LBSN, and the aggregation into nascent fibers, was clearly demonstrated by electron microscopy of 4-week-old neointimae (Figure 8, A–C). It was further noted that in addition to the increased amount and aggregation of mature elastin, LmBSN neointimae contained large amounts of immature and microfibrillar-rich elastin (Figure 8C), indicative of continuing elastin production and assembly. The elastin content of LBSN neointimae (Figure 8B) was generally lower than for the LXSN controls (Figure 8A), with much of the extracellular matrix occupied by matrix space and collagen fibrils. Generally collagen fibrils were more prominent in the LBSN neointimae than in

Figure 7. Orcein (dark red) staining of 2-week-old neointimae showing more abundant and more organized deposits of elastin in LmBSN compared with LXSN and LBSN seeded vessels. Elastin deposits in neointimae formed from mutant biglycan expressing cells are generally aggregated into bands, mostly parallel to the internal elastic lamina (**arrowhead**). Magnification = original $\times 300$.

LmBSN neointimae. A morphometric analysis, however, which showed a trend toward reduced collagen, did not reach significance. Morphometric analysis of the elastin

content in the mid to deep regions of the neointimae did confirm that neointimae of LmBSN seeded vessels contained significantly more elastin than either the LXSN or the

Figure 8. A, B, C: Electron micrographs of 4-week-old neointimae formed by LBSN (**A**), LBSN (**B**), and LmBSN (**C**); transduced cells show more abundant and aggregated mature elastin (**long arrows**). Microfibrillar-rich immature elastin (**short arrows**) in the extracellular matrix produced by mutant biglycan cells compared with matrix formed by vector alone cells (LXSN) and cells overexpressing normal human biglycan (LBSN). LBSN neointimae generally contained more collagen (co). Smooth muscle cells (SMC) are indicated. Magnification = original $\times 4000$. **D:** Volume fraction of elastin, determined by point counting of electron micrographs and expressed as a % of total intimal volume, showing significantly increased elastin in LmBSN neointimae and significantly reduced elastin in LBSN neointimae compared with LXSN.

LBSN neointimae, and that the elastin content of the LBSN neointimae was lower than LXS (Figure 8D).

Discussion

The results of this study show that biglycan is an important modulator of elastogenesis. Overexpression of biglycan core protein, mutated to prevent the attachment of the GAG chains, resulted in marked up-regulation of tropoelastin mRNA in vascular SMC and increased deposits of cross-linked and insoluble elastin *in vitro*, and *in vivo* in neointimae formed from GAG-deficient biglycan overexpressing cells seeded into balloon-injured carotid arteries. Overexpression of GAG-deficient biglycan also resulted in decreased synthesis and deposition of Type 1 collagen, thus shifting the balance in composition of the extracellular matrix. Conversely, overexpression of normal biglycan resulted in a decrease in elastic fiber deposition and an increase in collagen.

The mechanism by which overexpression of biglycan core protein leads to increased synthesis and deposition of elastin is not clear. Biglycan has been shown to bind, through its core protein, to both tropoelastin and to the microfibrillar component fibrillin,⁶ and increased expression of biglycan correlates with the elastogenic phase of elastic fiber formation during development.²⁷ These findings support the concept that biglycan may play a role in elastogenesis by promoting the interaction of the components involved in elastic fiber assembly. The findings of this present study provide some support for that idea. Notably, fibulin-5 expression was significantly increased along with the increase in tropoelastin expression in the LmBSN cells, although the expression of another important component of the elastic fiber, fibrillin-1 was not increased compared with control cells. Lack of co-regulation of fibrillin-1 with tropoelastin, however, has been previously reported.²⁸

The findings in this present study also point to an inhibitory role for the GAG chains of biglycan in fiber assembly, a proposal that is supported by other recent studies on elastic fiber assembly. This inhibitory role of GAG-containing biglycan adds additional support for the previously proposed mechanism that a high local concentration of galactosugar-containing GAGs (chondroitin- and dermatan sulfates) interferes with elastic fiber assembly. The low elastogenic potential of LBSN SMC overexpressing the GAG-decorated biglycan, was similar to that previously reported in cultures in which the addition of versican, or CS chains alone, was shown to decrease elastic fiber assembly by vascular smooth muscle cells.^{1,4} This decrease was linked to decreased levels of elastin binding protein, the chaperone that delivers tropoelastin to the growing fiber. In the presence of CS or DS, tropoelastin is prematurely released from elastin binding protein leading to decreased assembly of elastic fibers at the cell surface.^{1,4,5,29} Conversely, as we previously reported, a low level of CS is permissive for elastogenesis. Knockdown of the large versican variants V0 and V1 and their constituent CS GAG chains by overexpression of versican antisense markedly stimulates elastic fiber formation *in vitro* and *in vivo*,⁵ and overexpression of V3, the splice variant of versican that lacks GAG chains, similarly markedly stimulates elastin synthesis and assembly.^{3,4} The

expression of a biglycan core protein that lacks GAGs argues for a similar involvement of galactose-containing GAG chains in the inhibition of elastogenesis.

In this present study, overexpression of normal biglycan decreased tropoelastin mRNA, elastic fiber assembly, and insoluble elastin. The close association of biglycan with cell surface components involved in elastogenesis likely facilitates the decrease in elastin. It has been known for some time that biglycan is a cell surface molecule³⁰ and more recent studies have shown that biglycan binds to both tropoelastin and fibrillin,⁶ which in turn are in close proximity to elastin binding protein on the cell surface. Biglycan also binds to other cell-associated matrix molecules including dystroglycan³¹ and collagen Type VI.^{32,33}

Whereas the absence of biglycan GAG chains was permissive for elastic fiber assembly, there was an opposing effect on collagen fiber deposition. *In vitro* there was a marked decrease in collagen content of cultures, and in neointimae formed by mutant biglycan-overexpressing cells, there was less collagen in the extracellular compartment, suggesting that the chains of biglycan are critical to collagen fiber assembly. Biglycan associates with Type I collagen and is considered to play a role in fibrillogenesis³⁴ and biglycan expression is increased in fibrotic conditions such as glomerulonephritis,³⁵ pulmonary,³⁶ and hepatic fibrosis.³⁷ Conversely, in abdominal aortic aneurysms, where there is disorganized collagen and loss of elastic fibers, biglycan expression is markedly decreased.⁸ Targeted disruption of biglycan leads to abnormal collagen fibrils and an osteoporosis-like phenotype³⁸ and recent studies show that this deletion leads to aortic dissection and rupture with altered collagen phenotypes and a suggestion of reduced collagen amounts.⁹ These studies collectively point to a role for biglycan in regulating collagen accumulation. The mechanism by which this occurs, however, awaits further study.

The ability to change the composition of the extracellular matrix of neointimae by manipulating the forms of biglycan expressed points to novel therapeutic approaches that may be applicable to a variety of pathological conditions. In elastin-deficient conditions, such as in atherosclerosis and aneurysms, promotion of elastogenesis may be possible by overexpression or application of core protein of biglycan. Similarly, with respect to collagen content, various fibrotic conditions may be ameliorated. Where there is a need for both elastin and collagen, as would likely be needed for effective repair of aneurysms, more sophisticated strategies may be required, but the results of this study point to biglycan as an important determinant of the composition of the extracellular matrix.

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