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Decorin and biglycan expression: its relation with endothelial heterogeneity

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Summary. Decorin and biglycan proteoglycans play important roles in the organization of the extracellular matrix, and in the regulation of cell adhesion and migration. Given morphological and functional endothelial heterogeneity, information is needed regarding whether endothelial cells (ECs) from different vascular beds possess different profiles of proteoglycan constituents of the basement membranes.

Here, we report that endothelia from different murine organs and EC lines derived thereof produce and secrete different patterns of proteoglycans. A faint colocalization between decorin and PECAM/CD31 was found on tissue sections from mouse heart, lung and kidney by immunofluorescence. Three EC lines derived from these organs produced decorin (100-kDa) and its core protein (45-kDa). Extracellular decorin recognition in culture supernatant was only possible after chondroitin lyase digestion suggesting that the core protein of secreted proteoglycan is more encrypted by glycosaminoglycans than the intracellular one. Heart and lung ECs were able to produce and release decorin. Kidney ECs synthesized the proteoglycan and its core protein but no secretion was detected in culture supernatants. Although biglycan production was recorded in all EC lines, secretion was almost undetectable, consistent with immunofluorescence results. In addition, no biglycan secretion was detected after EC growth supplement treatment, indicating that biglycan is synthesized, secreted and quickly degraded extracellularly by metalloproteinase-2. Low molecular-mass dermatan sulfate was the predominant glycosaminoglycan identified bound to the core protein. ECs from different vascular beds, with differences in morphology, physiology and cell biology show differences in the proteoglycan profile, extending their heterogeneity to potential differences in cell migration capacities.

Key words: Endothelial cells, Decorin, Biglycan, Glycosaminoglycans, Extracellular matrix

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Introduction

Proteoglycans are complex macromolecules that consist of one or more glycosaminoglycan (GAG) side chains, covalently bound to the core protein.

The small leucine-rich proteoglycans (SLRPs) are a family of extracellular matrix molecules characterized by the presence of tandem leucine-rich repeats, flanked on either side by clusters of conserved cysteine residues. Most members of the SLRP family can be divided into three subclasses, with Class I containing decorin, biglycan and asporin; Class II containing fibromodulin, luminican and kerotocan, among others; and Class III containing epiphycan/PG-Lb, mimecan/osteoglycin and opticin (Iozzo, 1998; Henry et al., 2001).

Decorin, the best characterized member of the growing family of Class I SLRPs, is a ubiquitous component of the connective extracellular matrix. Decorin is known to modulate the activity of growth factors, collagen fibrillogenesis, the activity of the tyrosine kinase receptor, angiogenesis, tissue remodeling, etc. (Hakkinen et al., 2000; Kuwaba et al., 2001; Zhu et al., 2005; Fiedler et al., 2008).

Decorin and biglycan are highly homologous in terms of their core protein structure. Depending on the tissue type, decorin core protein has a single dermatan or

chondroitin sulfate GAG attached near the N-terminus, at serine-4, and three N-linked oligosaccharides attached to the leucine-rich domain, while biglycan carries two GAG chains near the N-terminus and two N-linked oligosaccharides (Scott et al., 2004).

A differential regulation of decorin and biglycan as endothelial cells (ECs) migrate, proliferate or alter their phenotype has been reported (Kinsella et al., 1997, 2000; Nelimarkka et al., 1997). Besides, and despite the core protein homology described, decorin and biglycan can be diversely regulated at the site of injury by a finely balanced release of active cytokines (Kaji et al., 2004; Raines and Nicola, 2005; Tiedemann et al., 2005).

Migration and proliferation of ECs are critical events in the repair of injured vessels, in angiogenesis and vasculogenesis occurring at development, tumor growth and tissue repair. Neoangiogenesis in wounded cornea is reduced in the absence of decorin (Schönherr et al., 2004). Conversely, neoangiogenesis is enhanced during dermal wound healing (Järveläinen et al., 2006). These studies indicate a regulatory role of decorin in inflammation-associated angiogenesis.

On the basis of morphology, the microvascular endothelium in normal adult organisms has been divided into different phenotypes: continuous, fenestrated, and discontinuous (Risau, 1995). This heterogeneity of the endothelium is not clearly understood at the molecular levels, and little is known about whether SLRPs also present a heterogeneous production profile.

The aim of the present study was to analyze the expression of SLRPs decorin and biglycan in ECs from different vascular beds in order to evidence whether ECs heterogeneity comprises heterogeneous composition of these proteoglycans in their basement membranes.

Materials and methods

Dulbecco's modified Eagle medium (DMEM), sodium pyruvate and EDTA were purchased from Sigma-Aldrich, St. Louis, USA. Fetal bovine serum (FBS) was purchased from Natocor (Córdoba, Argentina). Antibodies included goat polyclonal antibody against human biglycan L-15 (sc-27936), rabbit polyclonal antibody against human decorin H-80 (sc-22753), from Santa Cruz Biotechnology, Inc., MEC 13.3 rat monoclonal IgG2a against PECAM-1/CD31 (550274) from Pharmingen and anti-ß actin (A 2228) from Sigma-Aldrich, St. Louis, USA.

Immunohistochemistry

Organs (lung, heart and kidney) from BALB/c male mice were frozen in liquid nitrogen and stored at -80°C. Cryostat sections (5-8 µm) were fixed in acetone for 10 min at room temperature and immunostained with anti-PECAM-1, anti-biglycan and anti-decorin antibodies. Cryostatic sections were preincubated with normal horse serum to prevent non-specific binding of secondary antibodies, following an overnight incubation with optimal dilutions of the primary antibody: (1:200), (1:100) and (1: 100), respectively. The slides were then incubated with FITC goat anti-rat IgG (1:45) (F0479, Dakocytomation), donkey anti-goat IgG-PE (1:100) (sc-3743, Santa Cruz Biotechnology, Inc.) and Alexa Fluor 546 goat anti-rabbit F_{ab2} (1:100) (A11071, Invitrogen). Immunofluorescence was evaluated with an Olympus FV 300 Confocal Microscope (model BX61), with Fluo View version 3.3 acquisition software provided by the manufacturer.

Cell lines and isolation of cellular fractions

The following EC lines were used for comparative analysis: H5V derived from polyoma middle T-transformed murine heart endothelium (Garlanda et al., 1994); 1G11, from microvascular lung endothelium (Dong et al, 1997) and REC-A4 isolated from renal tissue (Gazzaniga et al., 2004).

H5V cells were grown in DMEM supplemented with 10% FBS, while 1G11 and REC-A4 ECs were grown in DMEM supplemented with 20% FBS. Besides, culture media were supplemented with 1% non-essential amino acids, 2 mM sodium pyruvate, 100 mg/ml streptomycin and 100 UI/ml penicillin.

For SLRP detection and analysis, EC cultures were grown for 48 h to 80-90% confluence in complete medium to obtain conditioned supernatants.

As a control, H5V monolayers were stimulated with 30 µg/ml of EC growth supplement (ECGS) (Sigma, St Louis, USA) for 48 h and supernatants and cell fractions were collected as described below.

Cell lysates were prepared as described previously (Cataruzza et al., 2002). After lysis, cell extracts were centrifuged at 120 g for 5 min at 4°C. The resulting supernatant was centrifuged at 13,000 g for 30 min at 4°C to obtain the membrane fraction. The resulting pellet, containing subcellular compartments related to protein export, was resuspended in 10 mM Tris-HCl buffer, pH 7.2 and 1 mM $MgCl_2$, (chondroitin lyase buffer). Protein content in samples was measured with the BCA kit (Pierce, USA).

SLRP isolation from culture supernatants

Conditioned culture supernatant was applied to a DEAE-Sephadex column (18.0x1.5 cm) equilibrated with 8 M urea with 0.5% Triton X-100, 0.1 M Tris-HCl, pH 7.5 and 0.15 M NaCl. The column was washed and subjected to a linear gradient of 0.15-3 mol/L NaCl at a flow rate of 15 ml/h, and fractions were collected (Kinsella et al., 1997). The salt concentration was estimated by conductivity. Aliquots of eluted material were assayed by metachromasy produced by sulfated GAGs with 1,9-dimethyl methylene blue (Leta et al., 2002). Positive fractions were pooled, concentrated and precipitated with three volumes of absolute ethanol and dried. Dried samples were resuspended in the required buffer. Protein content in samples was measured as

described above.

Western blot

Decorin or biglycan core protein was detected by Western blot on SLRPs isolated from culture supernatants and membrane fractions. Samples digested or undigested with ABC chondroitinase were solubilized in SDS-containing sample buffer in the presence of ß-mercaptoethanol and resolved in 10% SDS-polyacrylamide gel electrophoresis (PAGE). The polyacrylamide gel was transferred to Invitrolon PVDF membranes (Invitrogen), blocked and exposed to primary antibodies (diluted 1:500 in blocking buffer) overnight at 4°C. Decorin or biglycan was detected using the avidin-biotin AB Complex/HRP (K 0355, Dako Cytomation) and diaminobenzidine substrate kits (SK-4100, Vector Laboratories Inc.). ß-actin detection was carried out as a loading control. Band densitometry was performed with the Gel-Pro 3.1 program.

Analysis of glycosaminoglycan chains recovered from the culture supernatants

SLRPs previously purified on a DEAE-Sephadex column were resuspended in 0.1M sodium acetate buffer, pH 5.0, containing 5 mM cysteine and 5 mM EDTA. Papain (1 mg) was added to the mixture (50 µg of total GAG determined by 1,9-dimethyl methylene blue) followed by an overnight incubation at 60°C with gentle agitation. The incubation mixture was centrifuged (2000g, for 20 min at room temperature). A 10%-cetylpyridinium chloride solution was added to the supernatant to a final concentration of 0.5%, and the mixture was left to stand at room temperature for 24 h. After centrifugation the GAG-cetylpyridinium complex, was dissolved in a solution of 2M NaCl/absolute ethanol (100:15 vol/vol), and the GAGs were precipitated with the addition of absolute ethanol. After 24 h the precipitates were collected by centrifugation. The final pellet, which constituted the total GAG preparation, was dried at 60ºC for 30 min and dissolved in distilled water (Tovar et al., 1998). GAGs were incubated with 0.02 units of heparinase (9025-39-2) (Sigma-Aldrich, St. Louis, USA) and chondroitin ABC (9024-13-9) (Sigma-Aldrich, St. Louis, USA) or AC lyase (9047-57-8) Sigma-Aldrich, St. Louis, USA) in Tris buffer, pH 8.0, for 3 h at 37°C. After incubation, sample digestion was stopped by freezing.

The composition and the molecular mass of GAG chains were estimated at 6% PAGE with or without chondroitin lyase pre-treatment. GAG samples (10 µg) were applied to a 0.75-mm thick polyacrylamide gel slab in 0.02 M sodium barbital buffer (pH 8.6). After electrophoresis (100 V for 30 min), the gel was stained with 0.1% toluidine blue in 1% acetic acid, and washed in 1% acetic acid. High molecular dextran (average molecular mass: 100-200 kDa) and dextran (average molecular mass: 64-76 kDa and 35-45 kDa) were used as molecular mass markers.

Zymogram for matrix metalloproteinases activity

Gelatinolytic activity of matrix metalloproteinases (MMPs) from culture supernatant was analyzed using gelatin zymography (Taraboletti et al., 2000). Serum-free samples in 70 mM Tris-HCl buffer (pH=6.8), 10% glycerol, 2% SDS and 0.01% bromophenol blue were applied to 10% polyacrylamide gels copolymerized with 1mg/ml gelatin. After electrophoresis, the gels were washed three times for 20 minutes with 2, 5% Triton-HCl, pH 7.5, 200 mM NaCl, and 5 mM $CaCl_2$. Gels were then stained with 0.5% Coomasie blue in methanol 25% and 10% acetic acid and destained in the same solution lacking Coomasie brillant blue R250 (Sigma-Aldrich, St Louis, USA). Supernatant from HT 1080 cells was used as a reference for human pro-MMP-9 and pro-MMP-2. Gelatinolytic activity was visualized by negative staining.

Statistical analysis

Data are expressed as mean ± SD. Graph Pad Prism 4 software program was used for statistical evaluation. The statistical analysis was performed with a two-way ANOVA and Bonferroni's test. Values of $p<0.05$ were considered significantly different from controls.

Results

EC SLRPs localization in heart, renal and lung tissues

We analyzed the presence of SLRP decorin in heart, renal and lung tissue sections by immunofluorescence. As can be observed in Figure 1, an intense decorin expression was found in striated heart muscle cells (Fig. 1B). Although PECAM/CD31 expression in ECs from murine heart was intense, we found a tenuous co-localization of decorin with this EC marker (Fig. 1B). Renal sections showed that the strongest decorin positivity was detected in renal tubules with a slightly lower intensity in the podocytes of the glomeruli (Fig. 1D). Renal EC expression of PECAM/CD31 was predominantly localized in glomeruli and a weak signal of co-localization between decorin and PECAM/CD31 was observed in these structures (Fig. 1D). Finally, the presence of decorin was diffused in alveolar pneumonocytes (Fig. 1E). PECAM/CD31 expression in lung sections was intense; however it was difficult to detect co-localization of decorin with this EC marker (Fig. 1E). When co-localization of biglycan with PECAM/CD31 in ECs was analyzed in heart, renal and lung tissue sections, no signal was detected (data not shown).

SLRP decorin in monolayer cultures of different endothelial cell lines

The weak signal of decorin in ECs prompted us to analyze if the proteoglycan expression could be traced on ECs lines derived from the organs previously

analyzed by immunofluorescence. The study was performed on conditioned supernatants and membrane fractions isolated from EC monolayer cultures by Western blotting, using the same specific antiserum employed in fluorescence analysis. Figure 2A shows the presence of the proteoglycan and its core protein

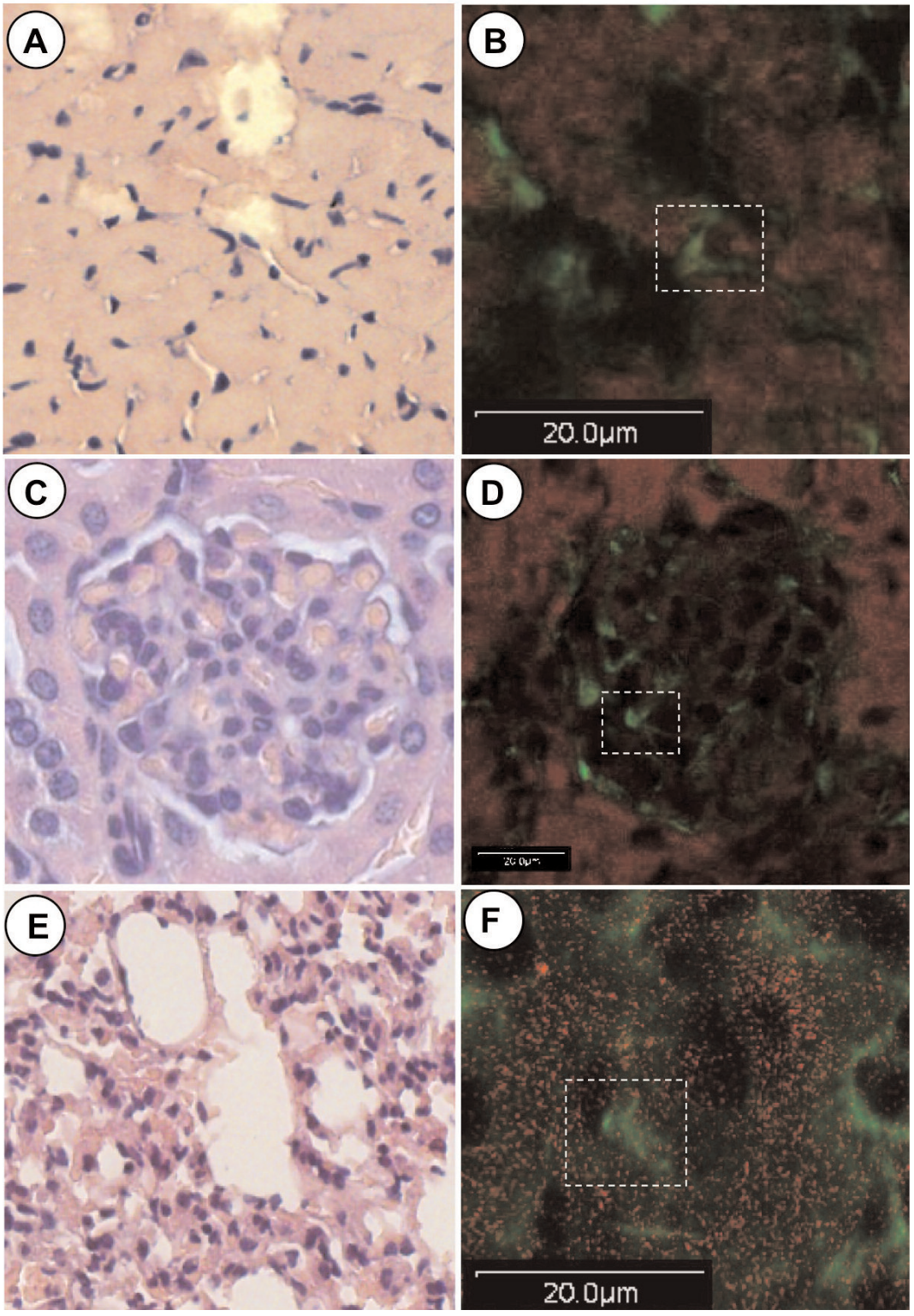

Fig. 1. ECs SLRPs localization in heart, renal and lung tissues. Serial sections of murine organs (heart, kidney and lung) were immunostained with anti-PECAM-1 and anti-decorin antibodies. **A.** Hematoxylin and eosin (H-E) staining of murine heart section. **B.** Staining of striated heart muscle cells with the anti-decorin antibody (red) and PECAM/CD31 expression (green) in ECs and a weak decorin co-localization with PECAM/CD31 in EC. **C.** H-E staining of murine renal glomerulus. **D.** Staining of glomerulus and tubules with the anti-decorin antibody and PECAM/CD31 expression in the endothelium of the glomerular capillaries with weak co-localization in EC. **E.** H-E staining of a murine lung section. **F.** A consecutive section showing lung parenchyma staining with anti-decorin antibody, specially in pneumocytes. PECAM/CD31 expression was intense in EC.

produced intracellularly. As can be seen, the three EC lines examined herein synthesized a proteoglycan with a molecular mass of around 100-kDa and its core protein, which migrated faster, with an average size of around 45-kDa. The quantification of the bands corresponding to the core protein showed no differences among the three ECs analyzed (Fig. 2B). Nevertheless, comparing the relative production of the core protein (45-kDa) to proteoglycan (100-kDa) for each EC line, we observed that renal (REC-A4) and lung (1G11) EC cultures produced around two times more core protein (45-kDa) ($P<0.01$) than decorin proteoglycan (100-kDa) (Fig. 2B). For heart (H5V) cells, similar amounts of both decorin core protein and proteoglycan were detected. These results could be attributable to different glycosylation rates of the protein core and/or different rate of proteoglycan secretion.

In order to evaluate decorin secretion, we analyzed the released proteoglycan in culture mediums by Western-blot. Decorin secretion was only evident for heart (H5V) and lung (1G11) EC lines (Fig. 2C). Chondroitin ABC digestion, which degraded chondroitin and dermatan sulfate, was needed to facilitate antibody recognition. In contrast, no band was detected for REC-A4 cells even after enzymatic treatment (Fig. 2C).

Although decorin proteoglycan production (100-kDa) of H5V cells was shown to have a higher level than 1G11 cells in membrane fraction (Fig. 2B), similar quantities were detected extracellularly (Fig. 2D).

In spite of the fact that a similar production of decorin core protein was detected for the three EC lines analyzed, different decorin proteoglycan production (100-kDa) and different extracellular detection were determined. Decorin production by renal ECs was evident but not detectable extracellularly. This result could be due to a very low decorin secretion rate or,

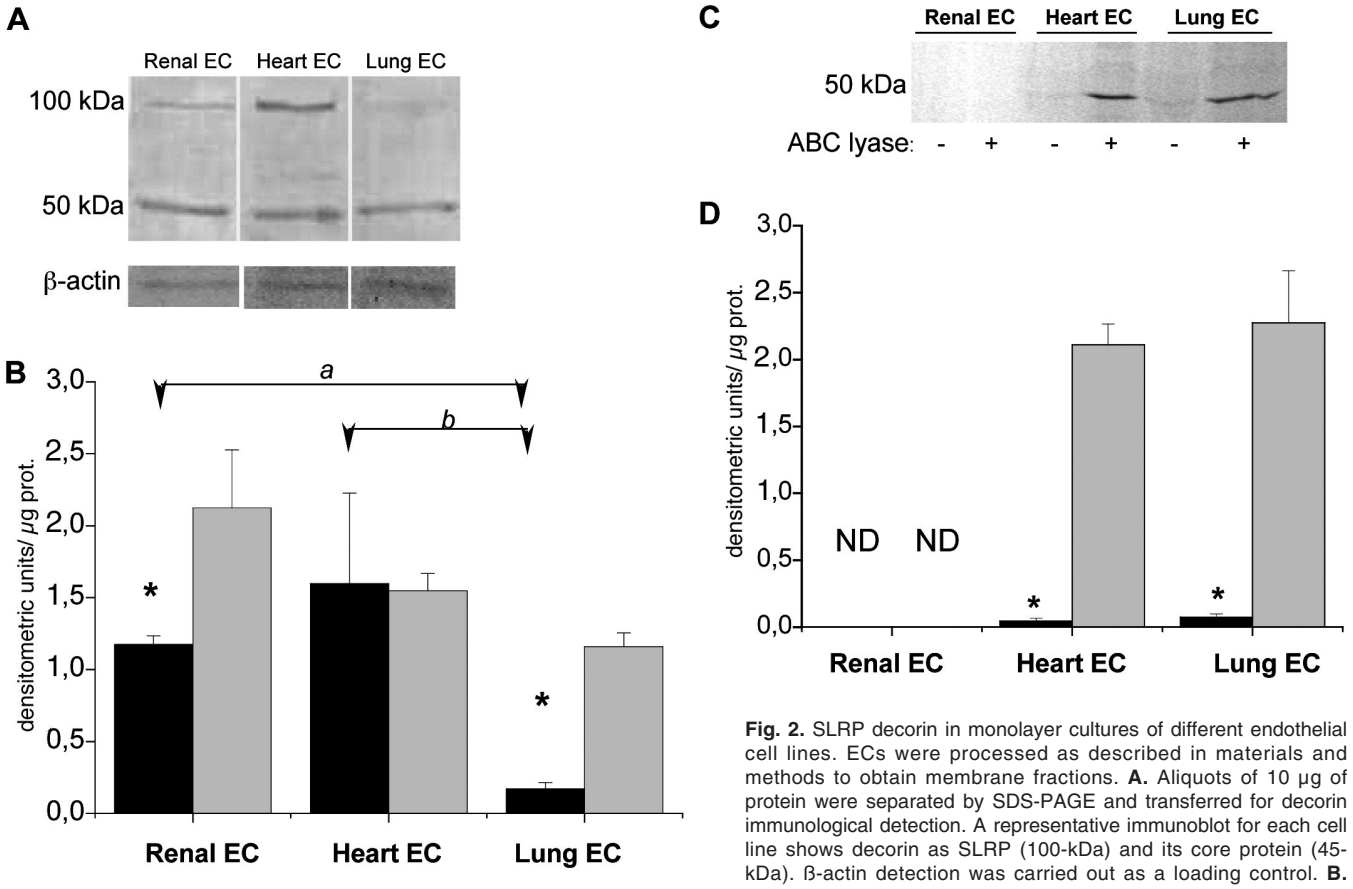

Fig. 2. SLRP decorin in monolayer cultures of different endothelial cell lines. ECs were processed as described in materials and methods to obtain membrane fractions. **A.** Aliquots of 10 µg of protein were separated by SDS-PAGE and transferred for decorin immunological detection. A representative immunoblot for each cell line shows decorin as SLRP (100-kDa) and its core protein (45-kDa). ß-actin detection was carried out as a loading control. **B.** Densitometric analysis of Western blots. Each column represents the relative amount of proteoglycan (black) and core protein (gray) normalized to protein content and expressed as arbitrary units/µg protein. The average of three independent experiments is expressed as mean ± SD (n=3) in each experiment. Significant differences in proteoglycan production between (a) Renal (REC-A4) vs Lung (1G11) ECs ($P<0.05$) and (b) Heart (H5V) vs Lung (1G11) ($P<0.01$) are shown. The asterisk indicates differences between protein core and proteoglycan. **C.** Decorin secretion was evaluated from culture supernatants recovered after 48 h (see methods). Samples were assayed before and after chondroitin ABC lyase digestion. **D.** Densitometric quantification is depicted, each column representing the relative amount of proteoglycan (black) and core protein (gray) normalized to protein content and expressed as arbitrary units/µg protein. Statistically significant differences ($P<0.001$) are indicated with an asterisk.

alternatively, to an extracellular enzymatic digestion.

SLRP biglycan in monolayer cultures of different endothelial cell lines

We detected biglycan by Western Blot on the murine EC lines: H5V, REC-A4 and 1G11. As Figure 3A shows, the three EC lines analyzed herein produced the proteoglycan (100-kDa) and its core protein (45-kDa).

Biglycan core protein (45-kDa) synthesis presented no differences between renal (REC-A4) and heart (H5V) ECs, while significant differences were observed between REC-A4 and 1G11 (P<0.001) and between H5V and 1G11 (P<0.001).

Biglycan proteoglycan (100-kDa) showed no significant differences between REC-A4 and H5V EC lines (Fig. 3B), in spite of a slight increment in the band corresponding to heart ECs (H5V). However, this proteoglycan expression presented significant differences when 1G11 was compared with REC-A4 (P<0.001) or H5V (P<0.001).

For every cell line, the results indicated that more than 1.5 times more core protein (45-kDa) than proteoglycan (100-kDa) were produced, suggesting a slow core protein glycosylation.

When biglycan was analyzed in the supernatants, no secretion was detected, despite chondroitinase treatment (data not shown). This result could be due to a low biglycan secretion rate or, alternatively, to an extracellular enzymatic digestion.

Biglycan production in stimulated H5V EC line

Considering the difficulty in extracellular biglycan detection, we decided to stimulate its production with ECGS in order to analyze a possible low rate of secretion. Heart EC line (H5V) was stimulated with 30 µg/ml ECGS. This stimulus produced a considerable increase in the yield of proteoglycan fractions isolated from conditioned supernatants, determined indirectly through protein content (0.5 mg/ml, without stimulus vs 24 mg/ml, with stimulus). As can be observed in Figure 4A, both the proteoglycan (100-kDa) (0.14 DU/µg of protein) and its core protein (45-kDa) (0.10 DU/µg of

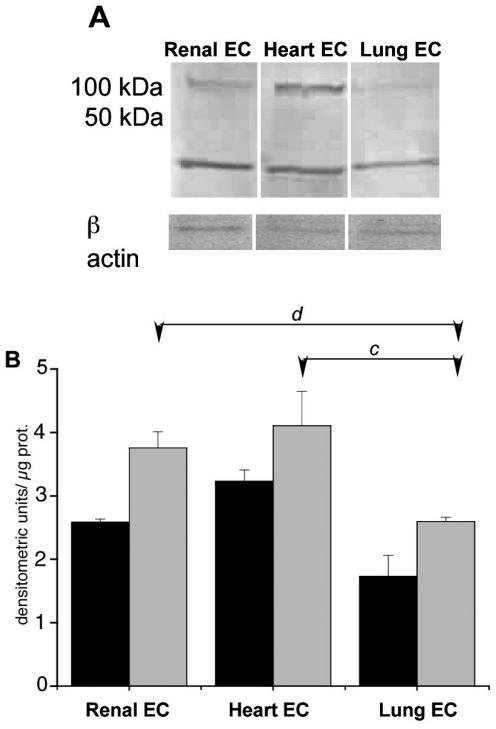

Fig. 3. SLRP biglycan in monolayer cultures of different endothelial cell lines. **A.** Aliquots of 10 µg of protein were separated by SDS-PAGE and transferred for biglycan immunological detection. A representative immunoblot for each cell line shows biglycan as SLRP (100-kDa) and its core protein (45-kDa). ß-actin detection was carried out as a loading control. **B.** Densitometric analysis of Western blots. Each column represents the relative amount of proteoglycan (black) and core protein (gray) normalized to protein content and expressed as arbitrary units/µg protein. The average of three independent experiments is expressed as mean ± SD (n=3) in each experiment. Significant differences in core protein (45-kDa) production between (c) Renal (REC-A4) vs Lung (1G11) ECs (P<0.001) and (d) Heart (H5V) vs Lung (1G11) (P<0.001) are indicated. Each EC line produces more than 1.5 times more core protein (45-kDa) than proteoglycan (100-kDa).

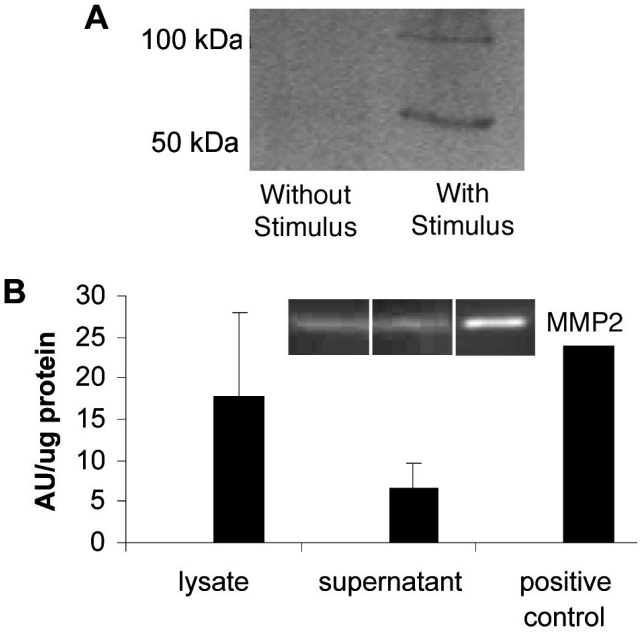

Fig. 4. Biglycan production in stimulated H5V EC line. **A.** Aliquots of 100 µg of protein were separated by SDS-PAGE and transferred for biglycan immunological detection. A representative immunoblot of the supernatant obtained from H5V EC cultures and ECGS-stimulated H5V is shown. **B.** Zymographic analysis: Supernatants were collected from heart (H5V) ECs. MMP activity in the culture supernatants was determined by zymogram. The zymogram was scanned and the intensity of the bands was quantitied. Supernatant of HT 1080 was used as a reference standard for human pro-MMP-2 and 9.

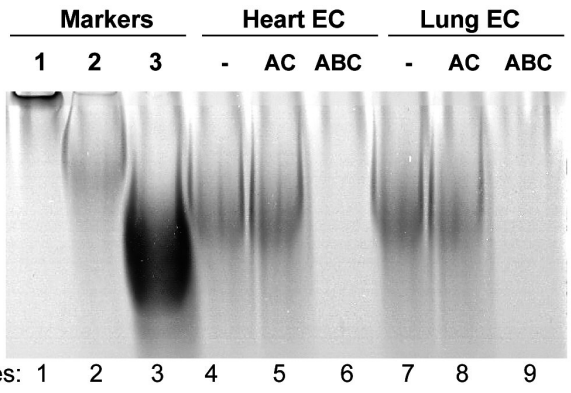

Fig. 5. Identification of GAGs. Electrophoretic identification of the GAG fraction obtained after enzymatic degradation. Lanes 1, 2 and 3 correspond to molecular mass markers with an average molecular mass of 100-200 kDa, 64-76 kDa and 35-45 kDa, respectively. The SLRP fractions obtained from H5V-conditioned supernatants were untreated (Lane 4), treated with lyase AC (Lane 5) or treated with lyase ABC (Lane 6). An identical scheme of seeding was used for 1G11 (lanes 7, 8 and 9).

protein) could be detected in the supernatant without the requirement of ABC enzymatic treatment. Considering that no enzymatic digestion was required, and that low band densities (DU/μg of protein) were detected in spite of the fact that around 10 times more protein was seeded, the results obtained suggested a rapid extracellular degradation of the proteoglycan. Therefore, zymographic studies were performed to characterize the presence of MMPs. Low-density cultures of H5V ECs were examined. The analysis revealed the production (cell lysate) and the secretion (supernatant) of MMP2 isoform of heart H5V cells (Fig. 4B).

Analysis of GAGs chains recovered from the culture supernatants

The identity and molecular size of GAG chains covalently bound to the core proteins were evaluated by PAGE on samples isolated from the culture supernatants treated with different mixtures of GAG lyases. As one of the chemical characteristics of GAG chains is their variable length, the analysis of electrophoretic results appears as broad bands corresponding to an average molecular mass (Fig. 5). GAG chains obtained after papain digestion of isolated SLRPs were pretreated with heparinase. Then, samples were incubated with AC or ABC chondroitinases, which degraded chondroitin sulfate or chondroitin and dermatan sulfate, respectively. No differences were detected for H5V either before or after AC chondroitin lyase digestion (Fig. 5, Lanes 4 and 5). On the other hand, no ABC chondroitin lyase-resistant fragments were obtained (Fig. 5, Lane 6), suggesting that H5V-secreted SRLPs were predominantly enriched in dermatan sulfate.

Similar results were obtained for the 1G11 EC line. (Fig. 5, Lanes 7, 8 and 9). This analysis could not be performed on REC-A4 supernatant as it was not possible to isolate proteoglycans from its culture mediums.

Consequently, dermatan sulfate with an average molecular mass of around 45-64 kDa was distinguished as the main GAG present in our endothelial SRLP samples.

Discussion

Microvascular endothelium in the normal adult organism has been divided into different phenotypes: continuous, fenestrated and discontinuous, regarding the morphology, physiology, cell biology, and biochemistry of the microvascular bed of each organ and tissue. All these different characteristics reflect potential differentiation pathways of ECs that have formed capillaries in a given organ or tissue by vasculogenesis or angiogenesis. However, adult endothelium, considered to be metabolically active but quiescent, must keep these phenotype characteristics during the entire life through interaction with the tissue environment, either by soluble factors or via cell-cell and cell-extracellular matrix interaction (Risau, 1995).

SLRPs are a family of extracellular molecules proposed to play roles in cell adhesion, growth factor interactions, and matrix assembly (Schönherr et al., 2005). This multiplicity of functions is mainly due to the unusual structure of the glycoprotein core, which harbors leucine-rich repeats flanked by cysteine-rich regions. The decorin core protein contains a single chondroitin/dermatan sulfate GAG, near the N-terminus, whereas biglycan carries two GAG chains, also localized near the N-terminus (Goldoni et al., 2004; Scott et al., 2006). The elucidation of the synthesis and secretion of class- I SLRPs gives new impetus to studies on their ligand binding and consequently to the understanding of their functions.

We report herein that endothelia from different murine organs such as kidney, heart and lung and EC lines derived thereof, produce and secrete different patterns of decorin and biglycan SLRPs. Our results demonstrate that decorin is expressed differentially in the endothelium of the lung, heart and kidney, stressing the concept of EC heterogeneity (Fig. 1). Moreover, this profile was conserved when we analyzed decorin produced and released by EC lines isolated from these organs. Thus, ECs in culture seem to maintain their differential capability to produce class-I SLRPs even when they are out of their microenvironment. This correlation has also been reported for other cell types, for example fibroblasts, for which *in vitro* culture did not affect decorin synthesis and production compared with primary isolates (Westergren-Thorsson et al., 2004; Honda and Munakata, 2004).

In our work, decorin core protein secreted to the medium of heart and lung EC cultures was only evident after chondroitinase ABC digestion (Fig. 2C). The requirement of an enzymatic treatment suggests that the

core protein of the extracellular decorin is more encrypted by GAGs than intracellular decorin. We performed these assays by using a polyclonal antibody raised against aminoacids 136-215 of a mammalian decorin. This sequence comprises from V to IX core protein domains, flanked by N-linked oligosaccharides (Goldoni et al., 2004). Since the position of the GAG chains seems to prevent antibody accessibility in secreted decorin, enzymatic digestion was required to favor antibody-epitope interaction.

The absence of decorin proteoglycan (100-kDa) in renal EC cultures could be the result of either a very low rate of the proteoglycan secretion or an extremely rapid degradation. EA.hy926 cells cultured in the presence of fibroblast or upon induction of decorin expression showed the upregulation of extracellular metalloproteinase MMP-1, MMP-2, 9 and the cell-associated MMP14, without any effect on MMP3 or on inhibitor of metalloproteinases 1 and 2 (TIMP-1 and TIMP-2) (Schönherr et al., 2001). Although this upregulation of proteases, an enhanced decorin degradation was not observed (Schönherr et al., 2001). In this study we have shown renal EC decorin proteoglycan (100-kDa) production (Fig. 2) and MMP-2 secretion (Fig. 4B). It is tempting to speculate that MMP-2 could be responsible for decorin degradation. Further studies will be required to address the issue.

Decorin involved in angiogenesis, particularly in association with a profound inflammation has been demonstrated in several studies (Nelimarkka et al., 2001; Burke et al., 2004; Fiedler et al., 2008). However, there have been conflicting reports of decorin as an angiogenesis inhibitor, especially through decorin derived antiangiogenic peptide LRR5 (Sulochana et al., 2005; Fan et al., 2008). We report here different patterns of decorin production by different EC lines which may correlate with different angiogenic capabilities related with the microenviroment.

Concerning biglycan, here we report that the proteoglycan (100-kDa) production for renal and heart ECs is similar, but differs from lung 1G11 cells, under the experimental conditions assayed.

As no extracellular biglycan detection was registered for any EC line analyzed, we evaluated the possibility of a rapid biglycan degradation after an ECGS stimulus (Fig. 4A). Our results suggest that the stimulus induced an increment in proteoglycan synthesis, followed by a rapid degradation process. These results are in accordance with those reported previously by Kinsella (Kinsella et al., 1997) where after bFGF treatment or wounding, the biglycan core protein is stimulated along with galactosaminoglycan chain synthesis. However, biglycan proteolytic processing also appears to be dramatically increased, with the result that the bulk of newly synthesized biglycan is rapidly cleaved after stimulation (Kinsella et al., 1997).

It is worth mentioning that the ECGS concentration used to stimulate ECs allows cell stimulation without inducing proliferation (Kinsella et al., 1997).

In the present study we show that the gelatinase, MMP2, is present and active in heart EC lines, both in cell lysate and in the supernatant (Fig. 4B). Recently, Augoff et al have reported the importance of TGF-ß liberation from its complex with decorin by activated MMP2 (Augoff et al., 2009). Proteoglycan degradation may be influenced by gelatinase accessibility to the core protein due to the glycosilation pattern. Here, we report that different amounts of decorin proteoglycan were detected in the media of the three ECs lines, associated with a different rate of glycosylation, and such differences may be responsible for an enhanced sensitivity to extracellular degradation.

GAG analysis demonstrated that both heart (H5V) and lung (1G11) ECs showed predominantly low-molecular-mass dermatan sulfate (Fig. 5). Different authors have described the importance of the GAG chain length, its sulfate degree and localization, properties that contribute to its great variety of biological functions (Tollfsen, 1995; Shirk et al., 2000; Calabrese et al., 2004; Alberto et al., 2008). Given that heart and lung ECs showed predominantly low-molecular-mass dermatan sulfate, differences in the sulfate content and/or localization could not be ruled out.

In summary, the EC lines from different vascular beds, cultured in the experimental setting here assayed were able to produce different quantities of decorin and biglycan. The EC lines examined herein synthesized both SLRPs with a molecular mass of around 100-kDa and its core protein (45-kDa). The ratio between these species showed a distinctly clear core protein accumulation for renal and lung endothelial cells. This protein accumulation could point out at different glycosylation rates and/or different rates of proteoglycan secretion among the three EC lines.

This work shows that EC lines from different vascular beds synthesize and secrete the SLRPs decorin and biglycan with heterogeneous profiles. In addition, we also provide indirect evidence of differences in core glycosylation. A more in-depth characterization of (a) SLRPs class I secretion and (b) aryl-sulphatases related with GAG polymerization mechanisms are required to better outline the SLRP pattern of expression associated with EC heterogeneity.

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