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TGF-β_1 generates a specific multicomponent extracellular matrix in human coronary SMC

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Abstract

Background Transforming growth factor (TGF-β_1) is postulated to play an important role in maintaining the structure and function of arterial tissue and protection against development of arteriosclerosis. The TGF-β_1-induced production of a stable extra-cellular matrix-rich plaque phenotype is suggested to be part of the protection against a switch to an unstable rupture-prone arteriosclerotic plaque.

Materials and methods This study addresses the question of whether the expression profile and the type of extra-cellular matrix (ECM) generated by TGF-β_1 stimulation have the structural feature of a fibril-rich stable matrix. Seventeen genes codings for ECM components of human coronary smooth muscle cells (SMCs) after a 24-h stimulation by TGF-β_1 have been analyzed.

Results Real-time RT-PCR was used to quantify the mRNA of genes under investigation. It was found that after TGF-β_1 stimulation (a) the up-regulation of COL1A1-specific mRNA was associated with increased [^{3}H]proline incorporation into the α-1 and -2 chains of collagen type I, (b) the up-regulation of biglycan- and syndecan-1-specific mRNA corresponded to an increased [^{35}S]sulphate and [4,5-^{3}H]leucine incorporation into the biglycan molecule and to an increase of syndecan-1 protein, (c) the up-regulated FGF-2 gene accounted predominantly for the ECM-bound subfraction of FGF-2-protein and (d) fibronectin and thrombospondin exhibited a significantly higher mRNA level. In contrast collagen XIV, a minor collagen type, and the proteoglycan decorin were down-regulated. The down-regulated decorin changed its structure by elongation and reduced GlcA to IdoA epimerization of the dermatan sulphate side-chain as judged by [^{35}S]sulphate metabolic labelling experiments. No significant changes in response to TGF-β_1 were observed for the collagen types III, VI and XVI, for versican, perlecan and the syndecans-2 and -4.

Conclusions It was concluded from the data that the TGF-β_1-induced formation of a highly specific multicomponent extra-cellular matrix on coronary arterial SMCs could provide *in vivo* mechanical strength to the neointima in arteriosclerotic lesions and to the fibrous cap overlying the lipid core.

Keywords Cytokines, extra-cellular matrix, fibrosis, gene analysis.
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Introduction

Arteriosclerosis is considered to be a chronic inflammatory disease. The balance between inflammation-induced degradation and newly synthesized deposition of extra-cellular matrix (ECM) components is thought to be an important factor for maintenance of plaque stability in humans [1,2]. The profibrotic activity of TGF-β_1 synthesized and secreted by vascular smooth muscle cells and macrophages would contribute to an increased stability of the neointima and to the mechanical strength of the fibrous cap overlying the lipid core of the plaque, thus promoting the transition from unstable rupture–prone to stable arteriosclerotic plaques [3,4]. Pleiotropic effects of TGF-β within the vasculature have been recently reviewed [2,3].

In cell cultures, exogenous TGF-β_1 stimulates the synthesis of collagen types I and III in human [5] and bovine [6] arterial smooth muscle cells (SMCs) and increases versican- and biglycan-specific mRNA in monkey arterial SMCs [7]. In

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addition, it has been shown that these two proteoglycans are localized in lipid-rich regions of arteriosclerotic plaques, along with TGF-β_1 [8], and exhibit an increased binding to low-density lipoproteins (LDL) owing to the longer glycosaminoglycan chains of these proteoglycans [9].

Evidence of an important role for TGF-β_1 in vascular disease has also been obtained from studies in experimental animals. Application of antisense oligodeoxynucleotides to TGF-β_1 significantly retarded balloon injury-induced neointima expansion [10], and mice heterozygous for the deletion of TGF-β_1 (TGF-β_1 +/–) exhibited an increased endothelial activation and lipid infiltration in the vascular wall [11]. Treatment of apolipoprotein E (apo E)-/– mice with a neutralizing antibody against TGF-β_1 developed arteriosclerotic lesions containing a high inflammatory cell content and a decreased amount of collagen [12].

Although numerous studies have shown that TGF-β increases collagen and proteoglycan deposition in the vasculature, a comprehensive insight into the ECM profile generated by TGF-β and its impact on plaque stabilization is still missing. This study investigated the effect of exogenous TGF-β_1 on the transcription of 17 genes encoding ECM components in human coronary SMCs. The TGF-β_1 was selected as the effector because the type I TGF-β receptor in SMCs responded dominantly to TGF-β_1 by elevating ECM production in diseased vessels [3,5]. The results provided evidence that TGF-β_1 generated a specific multicomponent matrix with the quality to contribute to the stability of arteriosclerotic plaques.

Materials and methods

Cell culture medium and SMC growth supplement were procured from TEBU-Bio GmbH (Offenbach, Germany). Tissue culture flasks were procured from Falcon Labware Division (Becton Dickinson & Co., Heidelberg, Germany). The [4,5–^{3}H]leucine (spec. activity 1·3 TBq mmol^{-1}), [2,3–^{3}H]proline (specific activity 1·74 TBq mmol^{-1}) and sodium[^{35}S]sulphate (carrier free, 0·8–1·5 TBq mg^{-1} sulphur) were obtained from Amersham Pharmacia Biotech (Freiburg, Germany).

Cell culture

Human coronary artery SMCs (HCASMCs) were produced by Cascade Biologics (Portland, OR) from tissue obtained from accredited US institutions. The cells were delivered by TEBU-Bio GmbH (Offenbach, Germany) as cryopreserved tertiary cultures with a certificate of analysis including positive tests for muscle α-actin compared with a known positive control culture. Cells were cultured as described previously [13], and 72 h before stimulation with TGF-β the standard medium was replaced by a minimum essential medium containing 0·5% fetal calf serum (FCS) (DMEM/FCS).

After preliminary time studies a 24-h stimulation by TGF-β_1 was found to be optimal and selected for the experiments. The TGF-β_1 effect was visible after 12 h and was present after 48 h. Items of the investigated ECM components were tested in parallel experiments by cryopreserved HCASMC from several donors certificated by the producers (Cascade Biologics or Clonetics, San Diego, CA). Cells of the 4th and 5th passages were used for the experiments. Cell proliferation was monitored by counting the number of cells, and total protein content was determined according to Lowry. Collagen was labelled for 24 h in DMEM/FCS containing 370 kBq mL^{-1} [2,3^{-3}H]proline. Sulphated glycosaminoglycans were labelled for 24 h in DMEM/FCS containing 370 kBq mL^{-1} [^{35}S]sulphate and processed as described [14]. The matrix-bound labelled collagen and sulphated proteoglycans were obtained as previously described [15]. Thereafter, the extra-cellular (subcellular) matrix was washed with phosphate buffered saline (PBS), digested with papain and analyzed for radioactivity. In some previous studies [13,14,23] it was found that under cell culture conditions HCASMC and normal vascular contractile SMCs undergo a gradual loss of myofilaments and a modulation to a synthetic phenotype. However, the cells still resemble SMCs under *in vivo* conditions – they contain α-actin and heavy chains of myosin and retained their capacity of extra-cellular matrix synthesis and the ability to respond to growth factors in a typical and reproducible manner.

RNA isolation and reverse transcription

Total cellular RNA was isolated from coronary SMCs using an RNeasy Mini kit (Qiagen, Hilden, Germany) as previously described [16]. Contaminating genomic DNA was removed using DNase I (Qiagen) and 5 μg of total RNA were reverse transcribed using 500 ng μL^{-1} oligo (dT) primer (Invitrogen, Karlsruhe, Germany), 1 mM dNTPs (Fermentas, St. Leon-Rot, Germany), 20 units of ribonuclease inhibitor (Promega, Mannheim, Germany) and 200 units of M-MuLV reverse transcriptase RevertAid (Fermentas) in a total volume of 20 μL. After diluting the cDNA 10-fold, aliquots were immediately used or stored at –20 °C.

Real-time RT-PCR

The PCR primers (Table 1) were designed using the Primer Express v2·0 software (Applied Biosystems, Weiterstadt, Germany) as previously described [17]. Primers were from Invitrogen and forward and reverse primers were located in different exons. Real-time RT-PCR were performed using the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) and the QuantiTect SYBR®Green PCR kit (Qiagen) as previously described [18]. Cycling parameters were an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Each reaction contained cDNA corresponding to between 0·2–7 ng total RNA and 200 nM of forward and reverse primer (Table 1). For each assay, cDNA samples were assayed in duplicate and analyzed using the Sequence Detection System v2·1 software (Applied Biosystems). Relative mRNA

Table 1 RT-PCR primers used in this study (forward and reverse primer are located in different exons)

Target mRNA	GenBank accession	Amplicon size (bp)	Forward primer	Reverse primer
SRP14	NM_003134	82	5′-AGCACTGTGGTGAGCTCCAAG-3′	5′-TCAGCCCATCCATGTTAGCTCTA-3′
GAPDH	NM_002046	115	5′-CAACAGCGACACCCACTCCT-3′	5′-CACCCTGTTGCTGTAGCCAAA-3′
COL1A1	NM_000088	61	5′-TACAGCGTCACTGTCGATGGC-3′	5′-TCAATCACTGTCTTGCCCCAG-3′
COL3A1	NM_000090	130	5′-AATTTGGTGTGGACGTTGGC-3′	5′-TTGTCGGTCACTTGCACTGG-3′
COL5A1	NM_000093	120	5′-GATTGAGCAGATGAAACGGCC-3′	5′-CCTTGGTTAGGATCGACCCAG-3′
COL6A1	NM_001848	146	5′-GACCTCGGACCTGTTGGGTAC-3′	5′-TACCCCATCTCCCCCTTCAC-3′
COL14A1	M64108	102	5′-TCCGAGGAATGGTATAACCGG-3′	5′-TGGACCAGGAACACTGACAGG-3′
COL16A1	NM_001856	84	5′-AGGCAAGGTCTACACCCGCT-3′	5′-TGGCTGATTCTTCCCGTCAG-3′
Biglycan	NM_001711	106	5′-GCCAAGCTGACTGGCATCC-3′	5′-AGTAGCGAAGCAGGTCCTCCA-3′
Decorin	NM_001920	101	5′-TGATGCAGCTAGCCTGAAAGG-3′	5′-AGGCGTGTTGGCCAGAGAG-3′
Fibronectin	NM_002026	123	5′-TGATCACATGGACGCCTGC-3′	5′-GAGTCAAGCCGGACACAACG-3′
FGF-2	NM_002006	125	5′-GATCCACCCCGACGGC-3′	5′-GCCAGGTAACGGTTAGCACACA-3′
Lumican	NM_002345	149	5′-TCATCCCTGGTTGAGCTGGAT-3′	5′-AGGATAATGGCCCCAGGATCT-3′
Perlecan	NM_005529	174	5′-TGTGTCGAGATGGAATCAAAGGA-3′	5′-GTCGGACTCTGCTATGCCATGT-3′
Syndecan-1	NM_002997	206	5′-ACAACTTCTCCGGCTCAGG-3′	5′-CACTTCTGGCAGGACTACAGC-3′
Syndecan-2	NM_002998	199	5′-GGAGTCGAGAGCAGAGCTGA-3′	5′-TGGAGCAGCACTAGTCAACA-3′
Syndecan-4	NM_002999	208	5′-GTCTGGCTCTGGAGATCTGG-3′	5′-CACCTTGTTGGACACATCCT-3′
Thrombospondin	NM_003246	108	5′-AGGCAAGGACTGCGTTGGT-3′	5′-GTACACTTCACGCCGGCAA-3′
Versican	NM_004685	128	5′-GTGGAAGGCACGGCAATCTA-3′	5′-TCTCCGCTGTATCCTGGCAC-3′

quantification was performed using the ΔΔCt method. The PCR efficiencies were calculated using cDNA dilutions derived from coronary SMCs and were taken into account for calculating fold changes which were finally normalized to the 14-kDa signal recognition particle (SRP14) or glycerolaldehyde-3-phosphate dehydrogenase (GAPDH). Following PCR amplification, samples were subjected to melting temperature analyses. The identity of PCR products was verified by sequencing as described in [17]. Controls containing all constituents, except the template, were performed to eliminate contamination of reagents.

Metabolic labelling of collagen type I

For *de novo* synthesis of fibrillar collagen cells were incubated with 370 kBq mL^{-1} [2,3^{-3}H]proline for 24 h in the presence or absence of 10 ng mL^{-1} TGF-β_1 [13,15] followed by exhaustive pepsin digestion. The pepsin digest was separated on a 4–12·5% gradient SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) under reducing conditions. The resolved α-1 and α-2 chains were visualized by autoradiography.

Metabolic labelling of decorin and biglycan

For *de novo* synthesis of the proteoglycans decorin and biglycan cells were labelled with 740 kBq mL^{-1} [4,5–^{3}H]leucine for 24 h in the presence or absence of 10 ng mL^{-1} TGF-β_1. The medium was processed for biglycan and decorin according to references [15] and [18].

Chain lengths of decorin and biglycan glycosaminoglycans were determined after metabolic labelling with 370 kBq mL^{-1} [^{35}S]sulphate for 24 h followed by ammonium sulphate precipitation. Decorin and biglycan were separated as described previously. The precipitated decorin was eluted with 6 M guanidinium hydrochloride buffer and the supernatant was fractionated on a Superose 6 column (Amersham Pharmacia Biotech, Freiburg, Germany). The fractions of the precipitate (decorin) or supernatant (30–39, biglycan) were pooled and concentrated overnight with Aquazide (Calbiochem, Merck Biosciences GmbH, Schwalbach/Ts., Germany). The concentrated samples were dialyzed overnight to 10 mM sodium acetate. To remove heparan sulphate the samples were treated with 1 M of HNO_2 at pH 4. After neutralizing with NaOH the glycosaminoglycan chains from decorin and biglycan were subsequently released by a β-elimination reaction using 0·1 M of NaOH/1 M of $NaBH_4$ for 20 h at 37 °C. The mixture was neutralized with 50% acetic acid, and dialysed against water. After freeze-drying the samples were resolved in 6 M of guanidinium hydrochloride buffer and size fractionated on a Superose 6 column [19].

Epimerization of the glycosaminoglycan chains was determined on the fractionated glycosaminoglycan chains as described previously [20]. The disaccharides of the glycosaminoglycan chains from decorin and biglycan were obtained after chondroitin ABC lyase treatment and analyzed with Partisil Pac (Phenomenex, Aschaffenburg, Germany).

Syndecan analysis

Cell surface exposed syndecan ectodomains were identified according to a protocol described previously [21]. Sandwich immunoassays were designed for detection of the shed syndecan-1 and -4 ectodomains according to Schmidt *et al.* [22] using mouse monoclonal antibodies as capture, polyclonal rabbit antisyndecans as secondary antibodies and HRP (horseradish peroxidase)-conjugated goat antirabbit IgG (1 : 50 000) for colour development. The cell supernatant containing the shed ectodomains of syndecan-1 and -4 was freeze-dried, redissolved in a small volume, dialyzed and deglycosylated by heparitinase and chondroitin ABC lyase for an enhanced binding of antibodies.

FGF-2 enzyme immunoassay

The fibroblast growth factor-2 (FGF-2, basic fibroblast growth factor, bFGF) content of the cellular, pericellular and extra-cellular compartment was determined as previously described [23].

Statistics

Results were expressed as means ± SD of the specified number of experiments carried out on different cultures in duplicates or triplicates. Statistical significance was assessed using Student's paired *t*-test. $P \leq 0{\cdot}05$ was accepted as significant. Densitometric measurements of electrophoretic bands were carried out by the NIH image and analysis program.

Results

TGF-β_1-affected expression profile of genes coding for extra-cellular matrix components

Numerous earlier studies have indicated that TGF-β_1 is an active and central participant in the production of extracellular matrix [24]. The RT-PCR primers used in this study (Table 1) extended the spectrum of components involved in atheroprotection. In Fig. 1, the 17 components of the ECM investigated are classified according to collagen types, chondroitin sulphate/dermatan sulphate (CS/DS) proteoglycans, heparan sulphate (HS) proteoglycans and other structural components. The changes in mRNA content upon 24-h stimulation with 10 ng mL^{-1} TGF-β_1 culture medium were quantified for all components by real-time RT-PCR analysis compared with non-stimulated cells. An explorative analysis of the mRNA expression data revealed a different response on TGF-β_1 stimulation (Fig. 1). The response to TGF-β_1 of each parameter was investigated and confirmed several times (n = 3–5) through independent experiments both on RNA and protein level. Collagen type I was used as an internal standard in all RNA analyses. For the up-regulated genes a corresponding increase on the trans-

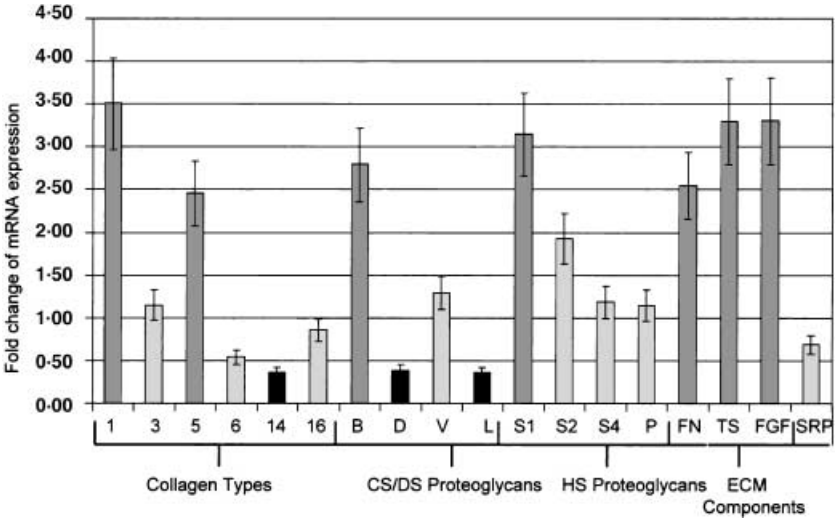

Figure 1 Transforming growth factor (TGF-β_1)-induced changes of the transcription of extra-cellular matrix-specific genes. Real-time RT-PCR analysis of mRNA coding for 17 genes of extra-cellular matrix components in response to TGF-β_1 stimulation was performed after incubating human coronary smooth muscle cells (SMCs) in the presence or absence (control) of 10 ng mL^{-1} TGF-β_1 for 24 h. B, biglycan; D, decorin; V, versican; L, lumican; S1, 2, 4, syndecans; P, perlecan; FN, fibronectin; TS, thrombospondin; FGF, fibroblast growth factor-2; SRP, signal recognition particle 14 kDa.

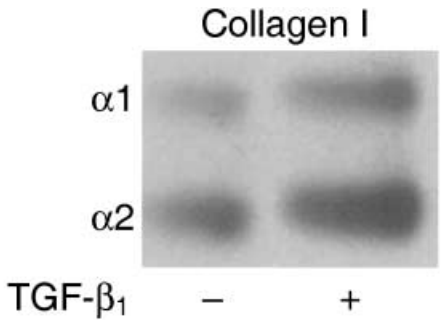

Figure 2 Polyacrylamide gel electrophoresis of collagen type I. Human coronary smooth muscle cells (SMCs) were metabolically labelled with 370 kBq mL^{-1} [2,3–^{3}H]proline in the presence or absence of transforming growth factor (TGF-β_1) (10 ng mL^{-1}) for 24 h. The α-1 and α-2 chains of collagen type I which accounts for > 90% of total collagen were clearly resolved and detected by autoradiography. Densitometry of bands from multiple experiments (n = 3) was carried out by the NIH image and analysis program.

lational level was secured by quantifying the corresponding proteins. Genes coding for extra-cellular components of vascular endothelial cells were not included in this study.

Collagens

Genes of the collagen type I and V α-1 chains (COL1A1 and COL5A1) showed a three-fourfold and a 2·5-fold increase, respectively, as shown in Fig. 1, but not that of the gene of the collagen type III, α-1 chain (COL3A1). The α-1 chain of all other collagen types exhibited no significant changes or a reduction (COL14A1) of the corresponding α-1 chain. The up-regulation of the COL1A1 gene (Fig. 1) corresponded to an over-expression of the α-1 and α-2 chains of collagen I and a significant increase in [^{3}H]proline incorporation into this collagen type. The PAGE analysis (Fig. 2) shows the α-1 and -2 chains of collagen I isolated from control and TGF-β_1-stimulated cells. The bands of α-1 and -2 chains of collagen type I obtained from three independent experiments were subjected to densitometry. The ratio of control:TGF-β_1 stimulation was found to be 1 : 2·7 for α-1 and 1 : 2·4 for α-2 chains ($P < 0{\cdot}05$). The increased amount of both α-chains after TGF-β_1 stimulation corresponded to an increase of cell protein from 2·57 μg 10^{-5} cells in controls to 4·87 μg 10^{-5} cells.

Type V collagen, a minor component of arterial collagen fibrils but implicated in the regulation of fibril diameter, was up-regulated after TGF-β stimulation.

It was found that the α-1 chain gene of collagen XIV was down-regulated by TGF-β_1 to < 50% of controls (Fig. 1) but its impact was not obvious as this collagen type contained several domains of other ECM components (see Discussion).

The elastin gene was not included in this study because the aortic elastin is known to be synthesized principally during perinatal and early growth and decreases to insignificant levels in the adult [25].

Proteoglycans

The protein cores of decorin and biglycan, two members of the small leucine-rich proteoglycans, are covalently linked with one or two dermatan sulphate chains. In coronary SMCs, biglycan is the dominant member of the small leucine-rich proteoglycans under physiological conditions. On the basis of an equal length of the PCR products of decorin (101 bp) and biglycan (106 bp) and an equal PCR efficiency, a ratio of the amounts of decorin and biglycan mRNA of approximately 1 : 9 in controls and 1 : 44 after TGF-β_1 treatment was found. As shown in Fig. 1, decorin and biglycan were differentially regulated and exerted an opposite effect in response to TGF-β_1. While biglycan showed a significant 2·5-fold increase of mRNA expression, decorin is down-regulated to < 0·5-fold. After metabolic labelling of the leucine-rich protein cores of decorin and biglycan with [^{3}H]leucine and deglycosylation of their glycosaminoglycan side chains, the effect of TGF-β_1 could be confirmed by identification of the

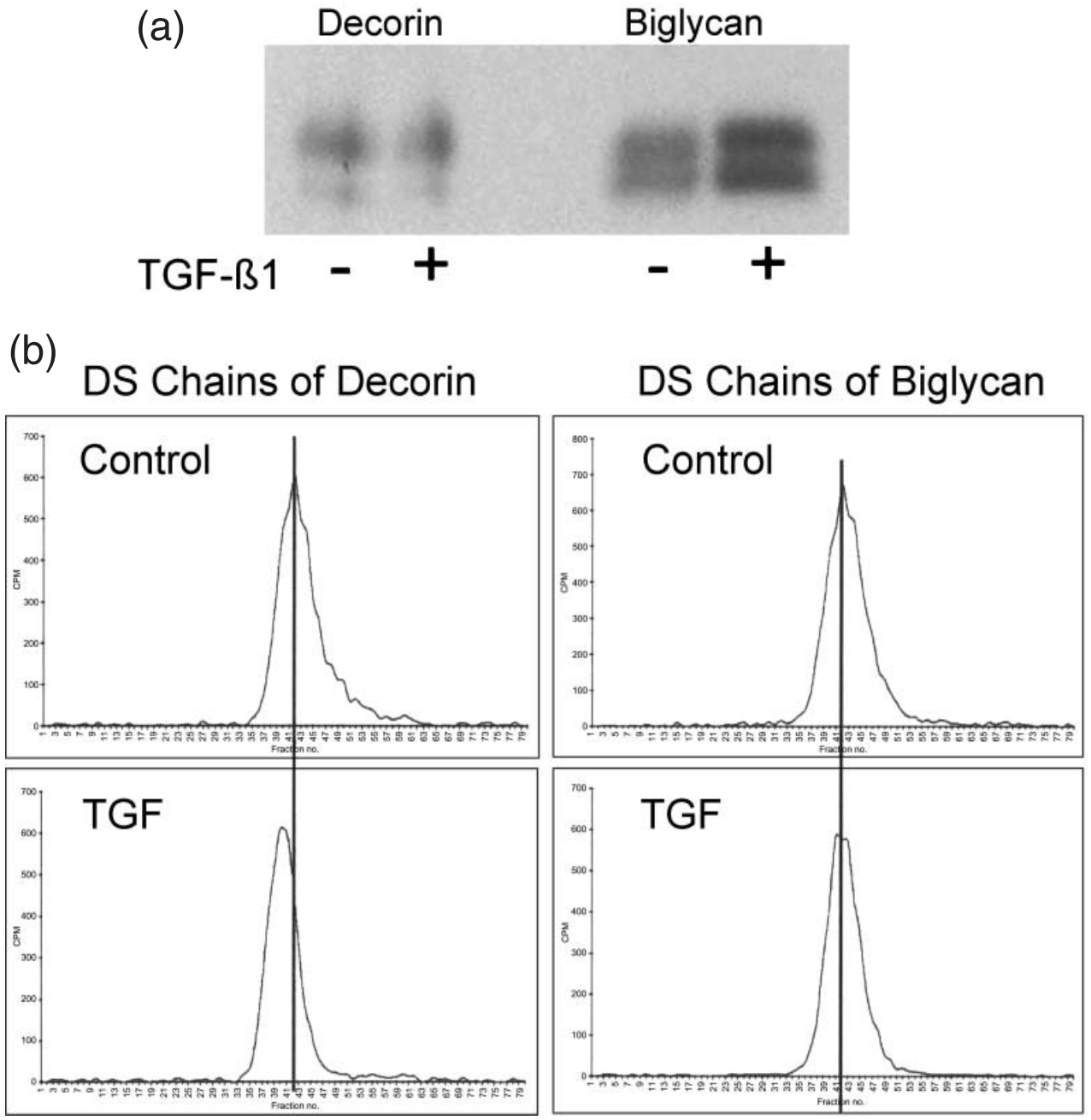

Figure 3 (a) *De novo* synthesis of decorin and biglycan protein core as influenced by transforming growth factor (TGF-β_1). Metabolically with 740 kBq mL^{-1} [4,5–^{3}H]leucine-labelled proteoglycans secreted from cells incubated in the presence or absence of TGF-β_1 (10 ng mL^{-1}) for 24 h were isolated, submitted to SDS-PAGE and visualized by autoradiography. Double bands (46 and 44 kDa) result from the presence of one or two N-glycosyl-oligosaccharide groups linked to the protein core. Densitometry of bands from multiple experiments (n = 3) were carried out by the NIH image and analysis program. (b) Determination of variations in the glycosaminoglycan chain length of decorin and biglycan. The [^{35}S] sulphate-labelled proteoglycans (370 kBq mL^{-1}) of human coronary artery smooth muscle cells (HCASMCs), incubated in the presence or absence of TGF-β_1 (10 ng mL^{-1}), were obtained from the culture medium after adsorption to a DEAE column and elution with 1 M NaCl. The dermatan sulphate chains from the protein core of decorin and biglycan were fractionated on a calibrated Superose 6 column. Control, dermatan sulphate chains of decorin and biglycan of normal or TGF, TGF-β_1-treated cells. The result is representative for three experiments.

newly synthesized [^{3}H]leucine-labelled protein core of decorin and biglycan by electrophoresis, as shown in Fig. 3a. Densitometric measurements of the decorin and biglycan double-bands revealed for the down-regulated decorin a ratio of control : TGF-β_1 stimulation of 1 : 0·78 and 1 : 0·69 and for the up-regulated biglycan of 1 : 3·2 and 1 : 12·3 ($P < 0·05$), respectively. The TGF-β_1-induced decrease of decorin was associated with structural changes of the molecule:

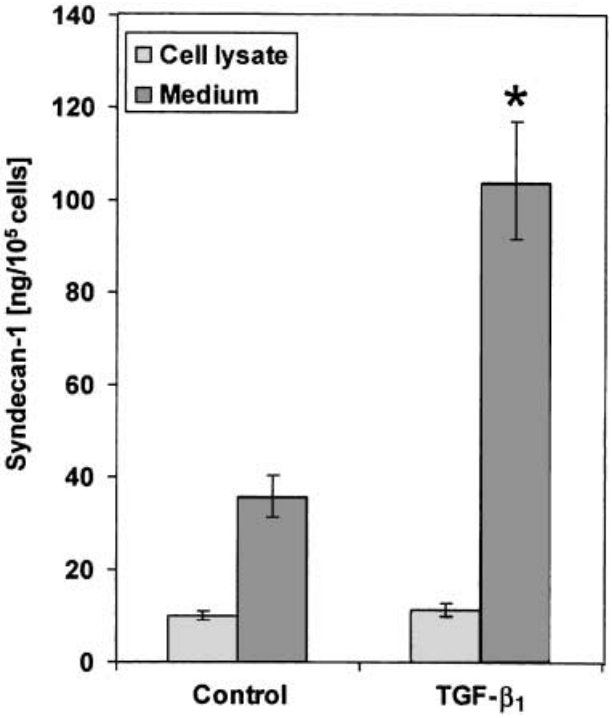

Figure 4 Syndecan-1 content of normal and transforming growth factor (TGF-β_1)-stimulated human coronary smooth muscle cells (SMCs). Immunoassays (ELISA) were made with cell lysates obtained after extraction with PBS containing 0·5% Brij^{35} (v/w) and with the culture medium ($n = 4$; $P < 0{\cdot}05$, normal versus TGF-β_1-stimulated cells).

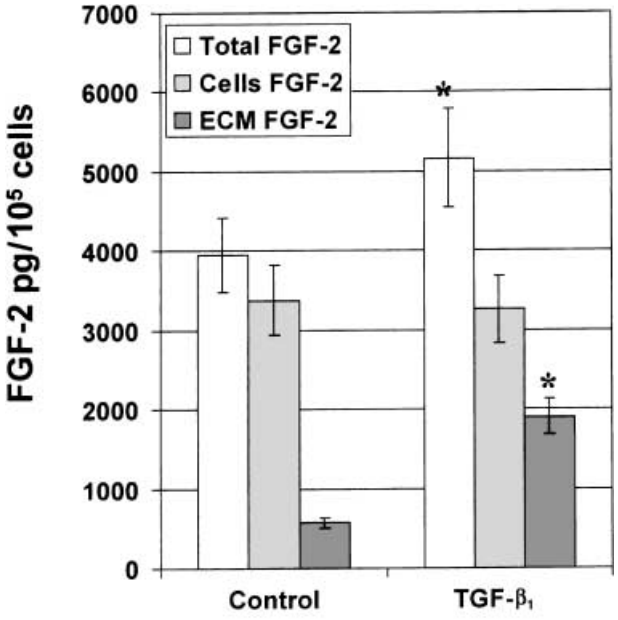

Figure 5 Transforming growth factor (TGF-β_1)-induced changes of FGF-2 concentration of human coronary smooth muscle cells (SMCs). Cells were incubated in DMEM/FCS in the presence or absence of TGF-β_1 (10 ng mL^{-1}) for 24 h. The synthesized total FGF-2 (bFGF) distributed to a cellular and extra(sub)cellular pool was quantified by an enzyme immunoassay as previously described [31] ($n = 3$; $P < 0{\cdot}05$, control total FGF-2/extra-cellular matrix (ECM) FGF-2 versus TGF-β_1 FGF-2/ECM FGF-2).

these concern an increased dermatan sulphate chain length, indicated by a smaller exclusion volume on Superose 6 chromatography (Fig. 3b), and a reduced GlcA to IdoA epimerization of the dermatan sulphate chain (not shown). The [^{3}H]GlcN/[^{35}S]sulphate double-labelling experiments did not indicate changes in the degree of sulphation of the glycosaminoglycan side chains of biglycan or decorin (not shown).

Syndecan-1 is a member of the four transmembranous heparan sulphate proteoglycans which are all expressed by vascular SMCs [26]. Syndecan-1 was up-regulated on stimulation with TGF-β_1 on the transcriptional and translational level as shown in Figs 1 and 4. Syndecan-4 content does not change in response to TGF-β_1 stimulation.

Fibroblast growth factor-2 (FGF-2, bFGF)

The functions of syndecan-1 chains include binding of the growth factor FGF-2. The TGF-β_1 causes an over-expression of FGF-2 (Fig. 5). In relation to the coincidental TGF-β_1-triggered enhanced synthesis of collagen I and V, on the one-hand, and FGF-2, on the other-hand, the syndecan-1 fulfils two main functions: (a) it promotes a close contact of SMCs to the newly synthesized ECM by interaction with collagen I, V and thrombospondin, and (b) the binding of the over-expressed FGF-2. The newly synthesized FGF-2 is known to be distributed to three different compartments [23]. As shown in Fig. 5, the bulk of total FGF-2 remains within the cellular and pericellular compartment while a small amount is deposited into the ECM where it binds in an inactive form to extra-cellular heparan sulphate. Under the influence of TGF-β_1 preferably and selectively the extra-cellular part of the FGF-2 increases significantly while the amount of cellular and pericellular FGF-2 remains nearly constant.

Under culture conditions vascular SMCs secrete the major part of ECM components in soluble form into the medium. Nevertheless, in electron microscopy, fibrillar ECM structures can be detected preferably in confluent cultures, but cross-striated collagen fibrils are not present. Hence, for an identification of the type and amount of produced ECM the analysis by molecular biological and biochemical methods appears to be more meaningful. Figure 6 shows the occurrence of extra-cellular fibrillar structures more frequently and to a higher extent in the extra-cellular space of TGF-β_1-stimulated cells.

Discussion

This study has shown that in SMC cultures the TGF-β_1 acted as a most potent stimulator of the synthesis of vascular extra-cellular matrix and induced the formation of a specific group of extra-cellular matrix components. Although previous

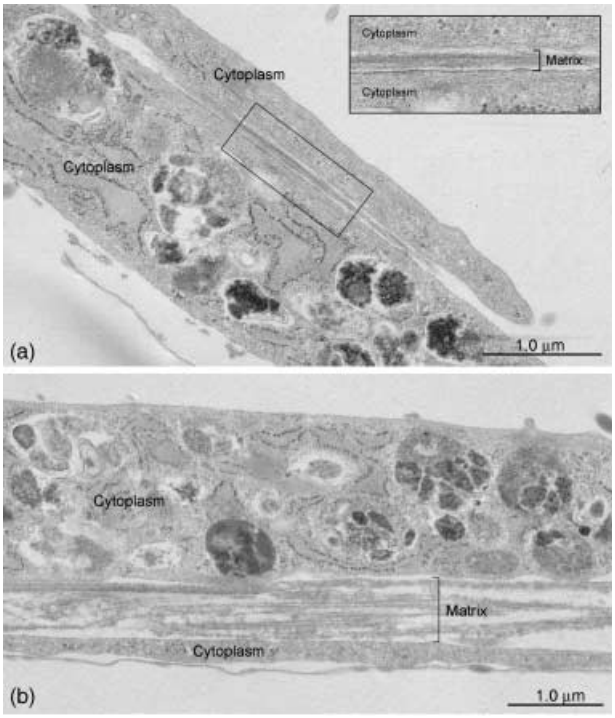

Figure 6 Electron micrographs of ultrathin vertical sections through hyperconfluent layers of human coronary artery smooth muscle cells (HCASMCs). Staining was with osmium tetroxide. (a) Control; fibrillar matrix in the extra-cellular space of adjacent cells is low (inset). (b) Increased fibrillar structures between two adjacent cells in TGF-β-stimulated cultures. The result is representative for multiple sections of three independent experiments.

investigators [27] have described a TGF-β_1-induced overproduction of collagen type I [28,29] and the occurrence of collagen I in the fibrous cap of primary plaques [30], the strength and the mechanical property of the fibrous cap is rather based on the formation of a stable fibril-rich matrix characterized by a proper assembly and structural integrity of selected ECM components rather than on overproduction of collagen. This should include the simultaneous up-regulation and expression of collagen and biglycan (Figs 1 and 3a), the up-regulation of collagen type V, the interaction of the transmembranous syndecan-1 with the ECM via the heparan sulphate chains of syndecan-1 and the heparan sulphate binding domains of fibronectin and thrombospondin (Figs 1 and 4). From the data it was concluded that TGF-β_1 could fulfil many of these desirable events and contributes to a stabilizing tendency of the newly synthesized matrix. Thus, TGF-β_1 leads not only to a mere overproduction of the vascular ECM but generates a TGF-β_1-specific matrix.

The prominent collagen types I and III are critical in coronary artery integrity [31], but the functions of other collagen types are less well understood. The up-regulated type V collagen is known to increase in arteriosclerotic plaques [32] and is thought to be implicated in the regulation of fibril diameter and is required for collagen fibril nucleation. The central role of collagen V in the regulation of fibrillogenesis is evident from studies on COL5A1$^+$-mice which show a reduced type V collagen content with a 50% reduction in fibril number and a subpopulation of large structurally abnormal collagen fibrils [33].

The α-1 chain of collagen XIV is down-regulated by TGF-β_1 but its impact is unclear. The complete primary structure of the mouse COL14A1 gene contains all structural domains described for a human. These include fibronectin type III repeats, von Willebrand factor A and TSP (thrombospondin) N-terminal-like domains [34].

Decorin and biglycan, the major proteoglycans of arterial tissue, exert an opposite effect in response to TGF-β_1. This result becomes more significant in view of the TGF-β_1-induced increase of COL1A1 and COL5A1 mRNA because the biglycan binds to collagen types I and V approximately as well as decorin [35], but the radio-labelled biglycan–collagen I interaction has a dissociation constant 100-fold greater than the radio-labelled decorin–collagen I interaction, which has a dissociation constant of 7×10^{-10} M [36]. Syndecan-1 binds SMCs via its heparan sulphate chains to a variety of ECM components and can serve as a matrix receptor. The ECM components bound by syndecan-1 include collagen types I, III, V, fibronectin, thrombospondin and tenascin [27], all of which are known to augment the anchoring of SMCs to the matrix.

The newly synthesized FGF-2 is known to be distributed to three different compartments [23]. The selective increase of the extra-cellular FGF-2 explains the missing mitogenic activity of the over-expressed FGF-2. However, the inactively bound FGF-2 forms a storage pool which could be released by heparitinase if a re-entry of SMCs into the cell cycle takes place [23]. Furthermore, the over-expressed FGF-2 could contribute to a controlled action of TGF-β because FGF-2 was reported to inhibit the TGF-β-stimulated collagen synthesis [37]. It was found that an elongation of the dermatan sulphate chains of decorin upon TGF-β_1 stimulation (Fig. 3b) would lead to an increased binding to LDL [38] and consequently to a lipoprotein accumulation in arteriosclerotic lesions. However, owing to the down-regulation of decorin synthesis in TGF-β_1-stimulated cells, this would probably be of no significance. Several studies [39] have demonstrated that extra-cellular matrix components have the capacity to bind lipoproteins. Proteoglycans and collagens have been shown to bind LDL and oxLDL. The negatively charged glycosaminoglycan chains on vascular proteoglycans interact with positively charged amino acids of apolipoprotein B and E leading to lipoprotein retention, a critical step in the initiation of atherosclerosis.

However, whether TGF-β_1 is operative and effective also in humans is still unknown although recent clinical studies, but not all [40], suggest a protective role of TGF-β in the process of plaque stabilization. Thus, carotid plaques from asymptomatic coronary heart disease (CHD) patients obtained by endarterectomy contained up to threefold more TGF-β, as demonstrated by increased TGF-β protein expression as compared with plaques from symptomatic CHD patients. Furthermore, plaques from the asymptomatic group had fewer macrophages and T-lymphocytes

than symptomatic plaques [41]. Patients suffering from unstable angina pectoris exhibited decreased levels of plasma TGF-β indicating a correlation of TGF-β levels with advanced arteriosclerosis [42]. In patients with proven coronary artery disease (CAD) a group with low plasma levels of TGF-β_1 had a significantly worse prognosis in terms of survival as compared with a group with high levels of plasma TGF-β_1, suggesting that the plasma concentration of TGF-β_1 may have a prognostic significance in patients with CAD [43].

The present data provide additional evidence for a plaque-stabilizing property of TGF-β_1. First, TGF-β_1 in general induced the expression of ECM components. Second, the profile of ECM components over-expressed upon TGF-β_1 stimulation of human coronary SCMs appears to be equivalent to an extra-cellular matrix with the quality to strengthen the mechanical properties of the fibrous cap and the ability to revert an unstable to a stable arteriosclerotic plaque. Conversely, a loss of ECM synthesis, a predominant enzymatic degradation of ECM or the presence of TGF-β inhibitors would convert a stable to an unstable plaque.

Further studies focused on the molecular organization and metabolic control of extra-cellular matrix turnover are necessary to better understand the pathogenesis of arteriosclerotic plaques and to establish a selective therapy.

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References

1 Ross R. Atherosclerosis – an inflammatory disease. *N Engl J Med* 1999;**340**:115–26.
2 Hansson GK. Mechanisms of disease: Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005;**352**:1685–95.
3 Grainger DJ. Transforming growth factor-β and atherosclerosis: so far, so good for the protective cytokine hypothesis. *Arterioscler Thromb Vasc Biol* 2004;**24**:399–404.
4 Lutgens E, Gijbels M, Smook M, Heeringa P, Gotwals P, Koteliansky VE *et al.* Transforming growth factor-β mediates balance between inflammation and fibrosis during plaque progression. *Arterioscler Thromb Vasc Biol* 2002;**22**:975–82.
5 McCaffrey TA. TGF-betas and TGF-beta receptors in atherosclerosis. *Cytokine Growth Factor Rev* 2000;**11**:103–14.
6 Wight TN. Cell biology of arterial proteoglycans. *Arterosclerosis* 1989;**9**:1–20.
7 Schönherr E, Järveläinen HT, Kinsella MG, Sandell LJ, Wight TN. Platelet-derived growth factor and transforming growth factor-β_1 differentially affect the synthesis of biglycan and decorin by monkey arterial smooth muscle cells. *Arterioscler Thromb* 1993;**13**:1026–36.
8 Evanko SP, Raines EW, Ross R, Gold LI, Wight TN. Proteoglycan distribution in lesions of atherosclerosis depends on lesion severity, structural characteristics, and the proximity of platelet-derived growth factor and transforming growth factor-beta. *Am J Pathol* 1998;**152**:533–46.
9 Little PJ, Tannock L, Olin KL, Chait A, Wight TN. Proteoglycans synthesized by arterial smooth muscle cells in the presence of transforming growth factor-β_1 exhibit increased binding to LDLs. *Arterioscler Thromb Vasc Biol* 2002;**22**:55–60.
10 Merrilees MJ, Scott L. Antisense S-oligonucleotide against transforming growth factor-beta-1 inhibits proteoglycan synthesis in arterial wall. *J Vasc Res* 1994;**31**:322–9.
11 Grainger DJ, Mosedale DE, Metcalfe JC, Bottinger EP. Dietary fat and reduced levels of TGFbeta1 act synergistically to promote activation of the vascular endothelium and formation of lipid lesions. *J Cell Sci* 2000;**113**:2355–61.
12 Mallat Z, Gojova A, Marchiol-Fournigault C, Esposito B, Kamate C, Merval R *et al.* Inhibition of transforming growth factor-beta signaling accelerates atherosclerosis and induces an unstable plaque phenotype in mice. *Circ Res* 2001;**89**:930–4.
13 Schmidt A, Geigenmueller S, Voelker W, Seiler P, Buddecke E. Exogenous nitric oxide causes over-expression of TGF-β_1 and overproduction of extra-cellular matrix in human coronary smooth muscle cells. *Cardiovasc Res* 2003;**58**:671–8.
14 Schmidt A, Vlodavsky I, Völker W, Buddecke E. Differentiation of coronary smooth muscle cells to a cell cycle-arrested hypertrophic growth status by a synthetic non-toxic heparin-mimicking compound. *Atherosclerosis* 1999;**147**:387–97.
15 Nareyeck G, Seidler DG, Troyer D, Rauterberg J, Kresse H, Schönherr E. Differential interactions of decorin and decorin mutants with type I and type VI collagens. *Eur J Biochem* 2004;**271**:3389–98.
16 Stolle K, Schnoor M, Fuellen G, Spitzer M, Engel T, Spener F *et al.* Cloning, cellular localization, genomic organization, and tissue-specific expression of the TGFbeta1-inducible SMAP-5 gene. *Gene* 2005;**351**:119–30.
17 Stolle K, Weitkamp B, Rauterberg J, Lorkowski S, Cullen P. Laser microdissection-based analysis of mRNA expression in human coronary arteries with intimal thickening. *J Histochem Cytochem* 2004;**52**:1511–8.
18 Schaefer L, Beck KF, Raslik I, Walpen S, Mihalik D, Micegova M *et al.* Biglycan, a nitric oxide-regulated gene, affects adhesion, growth, and survival of mesangial cells. *J Biol Chem* 2003;**278**:26227–37.
19 Zamfir A, Seidler DG, Schönherr E, Kresse H, Peter-Katalinic J. On-line sheathless capillary electrophoresis/nanoelectrospray ionization-tandem mass spectrometry for the analysis of glycosaminoglycan oligosaccharides. *Electrophoresis* 2004;**25**:2010–6.
20 Seidler DG, Breuer E, Grande-Allen KJ, Hascall VC, Kresse H. Core protein dependence of epimerization of glucuronosyl residues in galactosaminoglycans. *J Biol Chem* 2002;**277**:42409–16.
21 Schmidt A, Goepfert C, Feitsma K, Buddecke E. Lovastatin-stimulated superinduction of E-selectin, ICAM-1 and VCAM-1 in TNF-α activated human vascular endothelial cells. *Atherosclerosis* 2002;**164**:57–64.
22 Schmidt A, Echtermeyer F, Alozie A, Brands K, Buddecke E. Plasmin- and thrombin-accelerated shedding of syndecan-4 ectodomain generates cleavage sites at Lys^{114}-Arg^{115} and Lys^{129}-Val^{130} bonds. *J Biol Chem* 2005;**280**:34441–6.
23 Schmidt A, Skaletz-Rorowski A, Breithardt G, Buddecke E. Growth status- dependent changes of bFGF compartmentalization and heparan sulfate oligosaccharide pattern in arterial smooth muscle cells. *Eur J Cell Biol* 1995;**67**:130–5.

24 McCaffrey TA, Consigli S, Du B, Falcone DJ, Sanborn TA, Spokojny AM, Bush HL Jr. Decreased type II/type I TGF-beta receptor ratio in cells derived from human atherosclerotic lesions. Conversion from an antiproliferative to profibrotic response to TGF-beta1. *J Clin Invest* 1995;**96**:2667–75.
25 Wight TN. The vascular extra-cellular matrix. In: Fuster V, Ross R, Topol EJ, eds. *Atherosclerosis and Coronary Artery Disease*. Philadelphia: Lippincott-Raven;1996,pp.421–40.
26 Mertens G, Cassiman JJ, Van den Berghe V, Vermylen J, David G. Cell surface heparan sulfate proteoglycans from human vascular endothelial cells. Core protein characterization and antithrombin III binding properties. *J Biol Chem* 1992;**267**:20435–43.
27 Bernfield M, Götte M, Park PW, Reizes O, Fitzgerald ML, Linceum J *et al.* Functions of cell surface heparan sulfate proteoglycans. *Annu Rev Biochem* 1999;**68**:729–77.
28 Rekhter MD. Collagen synthesis in atherosclerosis: too much and not enough. *Cardiovasc Res* 1999;**41**:376–84.
29 Amento EP, Ehsani N, Palmer H, Libby P. Cytokines and growth factors positively and negatively regulate interstitial collagen gene expression in human vascular smooth muscle cells. *Arterioscler Thromb* 1991;**11**:1223–30.
30 Jimi S, Sakata N, Matunaga A, Takebayashi S. Low density lipoproteins bind more to type I and III collagens by negative-charge dependent mechanisms than to type IV and V collagens. *Atherosclerosis* 1994;**107**:109–16.
31 Riessen R, Isner JM, Blessing E, Loushin C, Nikol S, Wight TN. Regional differences in the distribution of the proteoglycans biglycan and decorin in the extra-cellular matrix of atherosclerotic and restenotic human coronary arteries. *Am J Pathol* 1994;**144**:962–74.
32 Ooshima A. Collagen β chain: Increased proportion in human atherosclerosis. *Science* 1981;**213**:666–8.
33 Wenstrup RJ, Florer JB, Brunskill EW, Bell SM, Chervoneva I, Birk DE. Type V collagen controls the initiation of collagen fibril assembly. *J Biol Chem* 2004;**279**:53331–7.
34 Gerecke DR, Meng X, Liu B, Birk DE. Complete primary structure and genomic organization of the mouse COL14A1 gene. *Matrix Biol* 2003;**22**:209–16.
35 Hocking AM, Strugnell RA, Ramamurthy P, McQuillan DJ. Eukaryotic expression of recombinant biglycan. Post-translational processing and the importance of secondary structure for biological activity. *J Biol Chem* 1996;**271**:19571–7.
36 Schönherr E, Witsch-Prehm P, Harrach B, Robenek H, Rauterberg J, Kresse H. Interaction of biglycan with type I collagen. *J Biol Chem* 1995;**270**:2776–83.
37 Davidson JM, Zoia O, Liu JM. Modulation of transforming growth factor-beta-1 stimulated elastin and collagen production and proliferation in porcine vascular smooth muscle cells and skin fibroblasts by basic fibroblast growth factor, transforming growth factor-alpha, and insulin-like growth factor-I. *J Cell Physiol* 1993;**155**:149–56.
38 Meyers CD, Tannock LR, Wight TN, Chait A. Statin-exposed vascular smooth muscle cells secrete proteoglycans with decreased binding affinity for LDL. *J Lipid Res* 2003;**44**:2152–60.
39 Chait A, Wight TN. Interaction of native and modified low-density lipoproteins with extra-cellular matrix. *Curr Opin Lipidol* 2000;**11**:457–63.
40 Nikol S, Isner JM, Pickering JG, Kearney M, Leclerc G, Weir L. Expression of transforming growth factor-beta-1 is increased in human vascular restenosis lesions. *J Clin Invest* 1992;**90**:1582–92.
41 Grainger DJ, Kemp PR, Metcalfe JC, Liu AC, Lawn RM, Williams NR *et al.* The serum concentration of active transforming growth factor-beta is severely depressed in advanced atherosclerosis. *Nat Med* 1995;**1**:74–9.
42 Cipollone F, Fazia M, Mincione G, Iezzi A, Pini B, Cuccurullo C *et al.* Increased expression of transforming growth factor-beta-1 as a stabilizing factor in human atherosclerotic plaques. *Stroke* 2004;**35**:2253–7.
43 Tashiro H, Shimokawa H, Sadamatu K, Yamamoto K. Prognostic significance of plasma concentrations of transforming growth factor-beta in patients with coronary artery disease. *Coron Artery Dis* 2002;**13**:139–43.