

# Glycosaminoglycans modulate cell-matrix interactions of human fibroblasts and endothelial cells in vitro

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## SUMMARY

Contact of various cells with extracellular matrix molecules modulates their cellular functions and phenotype. Most investigations have employed dishes coated with purified matrix constituents or plain collagen I lattices omitting the effects of other important matrix components such as proteoglycans. In this study we analyze the effect of purified glycosaminoglycans (GAGs) on human fibroblasts and human umbilical vein endothelial cells (HUVEC) embedded within collagen I/III lattices.

HUVEC contracted collagen I/III gels far less efficiently than fibroblasts and addition of heparan sulfate and heparin almost completely inhibited contraction. In collagen gels HUVEC down-regulated collagenase mRNA while increasing collagen I, IV mRNA expression. Addition of heparin and heparan sulfate reversed the collagen IV mRNA induction whereas hyaluronic acid and chondroitin sulfate enhanced fibronectin and collagenase transcripts.

Fibroblasts readily contracted collagen gels, and mRNA levels for fibronectin, collagenase and interleukin-6 were stimulated. Gel contraction was mostly unaffected by the different glycosaminoglycans. Fibroblasts responded to the addition of dermatan sulfate, heparan sulfate and heparin with a decrease in fibronectin, collagenase and interleukin-6 mRNA. Binding studies revealed saturable binding sites on fibroblasts and HUVEC for <sup>35</sup>S-labelled heparin, demonstrating specificity for heparin and heparan sulfate over other GAGs in competition experiments.

This study implies that glycosaminoglycans participate in cell-matrix interactions by effectively modulating the cellular phenotype via high affinity binding sites.

Key words: Cell-matrix interaction, Glycosaminoglycan, Collagen, Fibroblast, Endothelial cell

## INTRODUCTION

Functions of human fibroblasts are substantially modulated by three-dimensional contact to extracellular matrix components in vitro (Coulomb et al., 1983; Nusgens et al., 1984; Mauch et al., 1988, 1989; Ruoslahti and Yamaguchi, 1991). When these cells are incorporated into a hydrated collagen gel they readily contract it to a dense tissue like structure (Bell et al., 1979). This process is dependent on cell density and collagen content within the dermal equivalent. Adherence of fibroblasts to the collagen gel is further enhanced by other matrix components such as fibronectin (Gillery et al., 1986). Collagen gel contraction is paralleled by a modulation of gene expression in human fibroblasts (Nusgens et al., 1984; Gillery et al., 1986; Mauch et al., 1988, 1989). Cellular proliferation, the collagen synthesis rate, collagenase gene expression and activation, as well as interleukin-6 synthesis, an inflammation associated cytokine, are all influenced in fibroblasts by the surrounding extracellular matrix (Nusgens et al., 1984; Mauch et al., 1989; Eckes et al., 1992). Further studies indicated that this modula-

tion requires contact of the cells to a three-dimensional support and also depends on specific adherence of the cells to the collagenous substrate via cell adhesion molecules.

Most information is available for the integrins which have been identified to be of outstanding importance for gel contraction as demonstrated by blocking experiments (Klein et al., 1991; Schiro et al., 1991). In addition, clustering of integrins by antibodies resulted in tyrosine phosphorylation of a 120 kDa protein indicating a role in signal transduction (reviewed by Zachary and Rozengurt, 1992).

Microvascular and human umbilical vein endothelial cells (HUVEC), too, respond to stimuli from the extracellular matrix (Montesano et al., 1983). Here, laminin and protease generated laminin fragments cause reorganization of monolayers of evenly dispersed endothelial cells into three-dimensional tubelike structures (Kubota et al., 1988; Grant et al., 1989). To date, however, little is known about metabolic consequences of three-dimensional matrix-contact versus monolayer cultures in endothelial cells.

All the previous studies utilized matrix preparations extracted

from various sources and of different compositions. Apart from collagens, proteoglycans are important as well as abundant constituents of connective tissues (reviewed by Ruoslahti, 1988). In addition, proteoglycans are not limited in their localization to the extracellular matrix. Several of them are also found anchored to the cell surface such as fibroglycan, serglycin, or syndecan (Oldberg et al., 1981; Marynen et al., 1989; Saunders et al., 1989). Proteoglycans contain covalently attached glycosaminoglycan side chains which are composed of variably sulfated modified N-acetylated disaccharide subunits. cDNA cloning of several core proteins has greatly helped understand the diversity of these proteins and their expression patterns (Bourdon et al., 1985; Krusius and Ruoslahti, 1986; Doege et al., 1987; Saunders et al., 1989; Zimmermann and Ruoslahti, 1989; David et al., 1992; Zimmermann et al., 1994). However, regulation of glycosaminoglycan synthesis and composition of glycosaminoglycan side chains of individual core proteins during proteoglycan synthesis are still poorly understood at present. Also, the effect of proteoglycans on cell-matrix interactions and the resulting phenotype as well as their influence on specific cellular functions are not characterized in detail (Guidry and Grinnell, 1987).

Here we analyze the impact of individual glycosaminoglycans (GAGs) on cell-matrix interactions in a three-dimensional system. For this purpose fibroblasts and HUVEC were embedded within a collagen I/III matrix with addition of various glycosaminoglycans. Our results demonstrate that gel contraction of fibroblasts is not influenced by glycosaminoglycans whereas heparan sulfate and heparin almost completely inhibit gel contraction by HUVEC cells. In fibroblasts and HUVEC individual glycosaminoglycans cause specific regulation of the cellular phenotype accompanied by marked modulation of steady-state mRNA levels for extracellular matrix proteins, collagenase and interleukin-6. Finally, specific binding sites can be demonstrated on both fibroblasts and HUVEC for heparan sulfate chains and heparin.

## MATERIALS AND METHODS

### Tissue culture

Normal human fibroblasts were obtained from skin explant cultures in DMEM (Gibco, Paisley, UK) containing 10% fetal calf serum (Gibco, Paisley, UK), 100 i.u./ml penicillin, 100 µg/ml streptomycin and 50 µg/ml ascorbic acid (all Sigma, Deisenhofen, Germany). Cells were expanded and large frozen stocks were prepared from two different strains after 2nd and 3rd passage. For experiments fibroblast cultures were initiated from the frozen stocks, expanded and used between passage 6-8.

Human endothelial cells were isolated from umbilical veins. Following several washes with PBS endothelial cells were released from the vessel wall by incubation with a solution containing 0.1% collagenase type I (Sigma, Deisenhofen, Germany) and 5% BSA (Serva, Heidelberg, Germany) in DMEM without FCS for 30 minutes. Liberated cells were pelleted by low speed centrifugation and propagated in RPMI 1640 (Gibco, Paisley, UK) containing 10% FCS and 2% pooled human serum obtained from third term pregnant women (pre-partum serum). This formulation supported substantial endothelial cell proliferation without adding purified growth factors. Prior to experiments endothelial cell cultures were shifted to RPMI 1640/10% FCS for 7 days with medium changes every 2-3 days to remove undefined growth factor activities present in the pre-partum serum. Endothelial cell identity was confirmed by the typical cobble-

stone morphology. IIF staining further demonstrated factor VIII related antigen expression (polyclonal rabbit anti-factor VIII related Antigen, Behring Werke, Marburg, Germany). Again frozen stocks were established from which endothelial cell cultures were derived.

### Glycosaminoglycan preparation

#### Chondroitin sulfate from bovine tracheal cartilage

Dry powder of bovine cartilage (1 g) was suspended in 12.5 ml papain buffer (0.1 M sodium acetate, 10 mM cystein hydrochloride, 10 mM EDTA, pH 6.5). Papain was added at 40 mg/ml and digestion was carried out at 65°C for 15 hours. After adjustment of the pH at room temperature to 6.5, cystein hydrochloride and papain were again added and digestion proceeded for another 15 hours at 65°C. The proteolysate was adjusted to pH 1.3 with 6 M HCl and kept at 4°C for 4 hours before being centrifuged at 10,000 rpm for 30 minutes at 4°C in a Spinco L2-65B rotor type 19. The supernatant was neutralized with 8 M NaOH and evaporated to 1/3 volume at 40°C. The solution was desalted by chromatography on a BioGel P2 column (equilibrated and eluted with 10% ethanol). The eluate was evaporated to a small volume and loaded on a Dowex 1×2 column equilibrated with 0.15 M NaCl. Elution was performed with 3 bed-volumes each of 0.15 M, 0.5 M, 1.25 M and 3.0 M NaCl. The fraction eluting at 1.25 M NaCl was desalted on a BioGel P2 column and evaporated to 1/3 starting volume. Since this fraction contained trace amounts of keratan sulfate the solution was loaded onto a cellulose column equilibrated with 1% cetylpyridiniumchloride (at 26°C). Elution was done with 3 bed-volumes of 1% cetylpyridiniumchloride, 50 mM MgCl<sub>2</sub> and 1 M NaCl solution. The last fraction was desalted and evaporated as above and the chondroitin sulfate was precipitated five times with sodium acetate saturated ethanol. The precipitate was dried in a vacuum desiccator. Analysis of the purified material revealed an average relative molecular mass of 18,000 Da, a sulfation degree of 0.92% (mol sulfate/mol GalN) and a disaccharide pattern of 51.3% (mol/100 mol) C4S-disaccharides, 40.9% C6S-disaccharides and 7.8% C0S-disaccharides.

#### Dermatan sulfate from porcine skin (high sulfation grade)

Porcine skin was defatted by acetone treatment and proteolyzed as detailed above. The crude fraction was loaded onto a Dowex 1×2 column which was eluted with 1.25 M NaCl. The elute was desalted, adjusted to 5% calcium acetate and fractionated by differential ethanol precipitation with 18%, 24%, 36% and 46% final ethanol concentrations. Precipitates from the 18%, 24% and 36% fractions were desalted and the calcium ions were removed by repeated precipitation with sodium acetate saturated ethanol. Since disaccharide patterns of these dermatan sulfate preparations were almost identical the three fractions were pooled. Analysis displayed a mean average relative molecular mass of 22,000 Da, a sulfation degree of 1.07 (mol sulfate/mol GalN) and a disaccharide pattern of 90.5% (mol/100 mol) DS-disaccharides, 7.1% 2-O-sulfo-idoUA-4-O-sulfo-GalNAc-units, 1.8% C4S-disaccharides and 0.6% C6S-disaccharides.

#### Heparan sulfate and heparin from bovine lung

Heparan sulfate and heparin were both provided partially purified from Upjohn (Kalamazoo, USA) as barium salts after β-elimination with NaOH. The GAGs were fractionated after replacement of barium ions for sodium by repeated precipitation. This solution was absorbed onto a Dowex 1×2 column and eluted with step gradients from 0.15 M-3.0 M NaCl. In the case of heparan sulfate, fractions were chosen eluting between 1.15 M and 1.75 M NaCl and for heparin eluting between 1.5 M and 2.0 M NaCl. The analysis data revealed a relative molecular mass of 8,000 Da.

GAGs were dissolved in 0.3 M sodium acetate buffer (pH 5.3) and subsequently concentrated by ethanol precipitation with 9 vols ethanol and centrifugation.

Hyaluronic acid prepared from human umbilical cord was purchased from Sigma (#H1751, Sigma, Deisenhofen, Germany). A

1 mg sample of the different GAGs was subjected to SDS-PAGE electrophoresis in order to detect contaminating proteins. However, Coomassie staining did not reveal any detectable banding pattern indicating the absence of impurities of the GAG preparations. Furthermore, enzymatic digestion of GAGs reversed biological effects observed with e.g. heparin (see below).

### Dermal equivalents

Dermal equivalents were prepared with a collagen solution from calf skin (Deutsche Gelatine Fabrik Stoess AG, Ebersberg, Germany) containing collagens type I and III. SDS gel electrophoresis demonstrated the typical banding pattern for collagen I and on retarded electrophoresis for collagen III. Collagen was purified by dialysis against low ionic strength, neutral pH buffer (20 mM sodium phosphate, pH 7.5). Precipitated material was collected by centrifugation, sterilized in 70% ethanol, freeze dried and redissolved in 0.1% acetic acid. The final concentration was adjusted to 3 mg/ml (stock solution). GAGs were added at a 0.2% concentration (collagen dry weight:GAG dry weight). For preparation of dermal equivalents 3 ml of the collagen:GAG solution were mixed on ice with 4.6 ml of a 1.76× DMEM salt solution, followed by neutralization with 1 M NaOH, and addition of 0.9 ml FCS; 10 ml DMEM containing  $1.8 \times 10^6$  cells/10% FCS were finally added. This solution was poured into 10 cm diameter dishes. Polymerization of the gels occurred at 37°C within 30 minutes and cells readily formed contacts to the extracellular matrix visible in phase contrast microscopy by small dendritic processes. Over 24 hours the cells contracted the gels and after 24 hours the diameter was measured. Total RNA was prepared from parallel cultures (see below). All experiments described were done twice with two different cell strains of fibroblasts and endothelial cells.

### LDH activity determination

Released lactate dehydrogenase [EC 1.1.1.27] activity in the medium was determined with a BM/Hitachi photometer using the LDH optical assay (Boehringer Mannheim, Mannheim, Germany).

### RNA isolation and hybridization

From dermal equivalents or monolayer cultures total RNA was isolated according to the method of Chomczynski and Sacchi (1987). Dermal equivalents were centrifuged prior to lysis in order to condense the lattices. Samples containing 5 µg total RNA were fractionated in a 1.2% agarose gel and transferred to nylon membranes (Hybond N, Amersham Buchler, Braunschweig, Germany). The transferred RNA was UV-crosslinked and hybridized to random primed cDNA probes (Feinberg and Vogelstein, 1983) for 18–24 hours according to the protocol of Church (Church and Gilbert 1984). The following hybridization probes were used in this study: procollagen  $\alpha 1(I)$  (Chu et al., 1982), collagen  $\alpha 1(IV)$ ,  $\alpha 2(IV)$  (Schwarz et al., 1986), fibronectin (Bernard et al., 1985), interstitial collagenase (kindly provided by Dr P. Angel, Karlsruhe, Germany), interleukin-6 (a gift from Dr L. T. May, New York, USA) and GAPDH (kindly provided by Dr J. Uitto, Philadelphia, USA) as an internal standard. After hybridization, filters were washed in 2× SSC (SSC: 0.15 M NaCl, 0.015 M trisodium citrate), 0.1% SDS, followed by 0.1× SSC, 0.1% SDS at 55°C and exposed at –70°C with intensifying screens.

### Binding assays

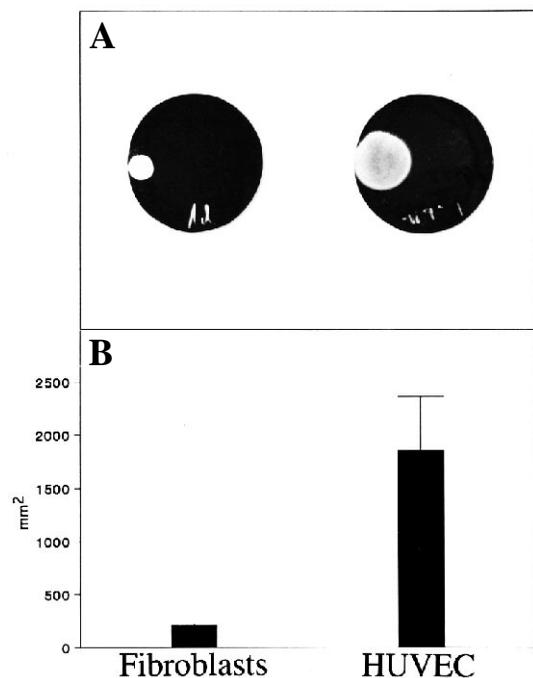
For binding assays fibroblasts or endothelial cells were cultured in 6-well plates (35 mm diameter) until confluence. Prior to the assay, medium was aspirated and cells were washed in binding medium (DMEM containing 1% BSA) two times at room temperature. Variable amounts of  $^{35}\text{S}$ -labelled heparin ( $[\text{Na-}^{35}\text{S-sulphonate}]$ heparin; Amersham Buchler, Braunschweig, Germany; specific activity 132.2 mCi/g) were incubated in binding medium for 1 hour at room temperature. Cells were then washed in binding medium three times; bound ligand was solubilized in 1% SDS and determined by scintillation counting. Non-specific binding was

measured in the presence of a 500-fold excess of unlabelled heparin (Sigma, Deisenhofen, Germany). For competition experiments 100 ng labelled ligand/ml was added to a variable excess of different unlabelled (cold) glycosaminoglycans in binding medium. This mixture was then incubated with endothelial cells and fibroblasts to determine competition of the different unlabelled GAGs for  $^{35}\text{S}$ -labelled heparin binding sites. The data were analyzed using weighted, non-linear least-squares curve fitting with the computer program LIGAND (Munson and Rodard, 1980). In addition, several transformed cell lines were analyzed accordingly to compare the binding data and calculated  $K_d$  values obtained here to previously published studies as an internal standard (Halper, 1990; Leung et al., 1989).

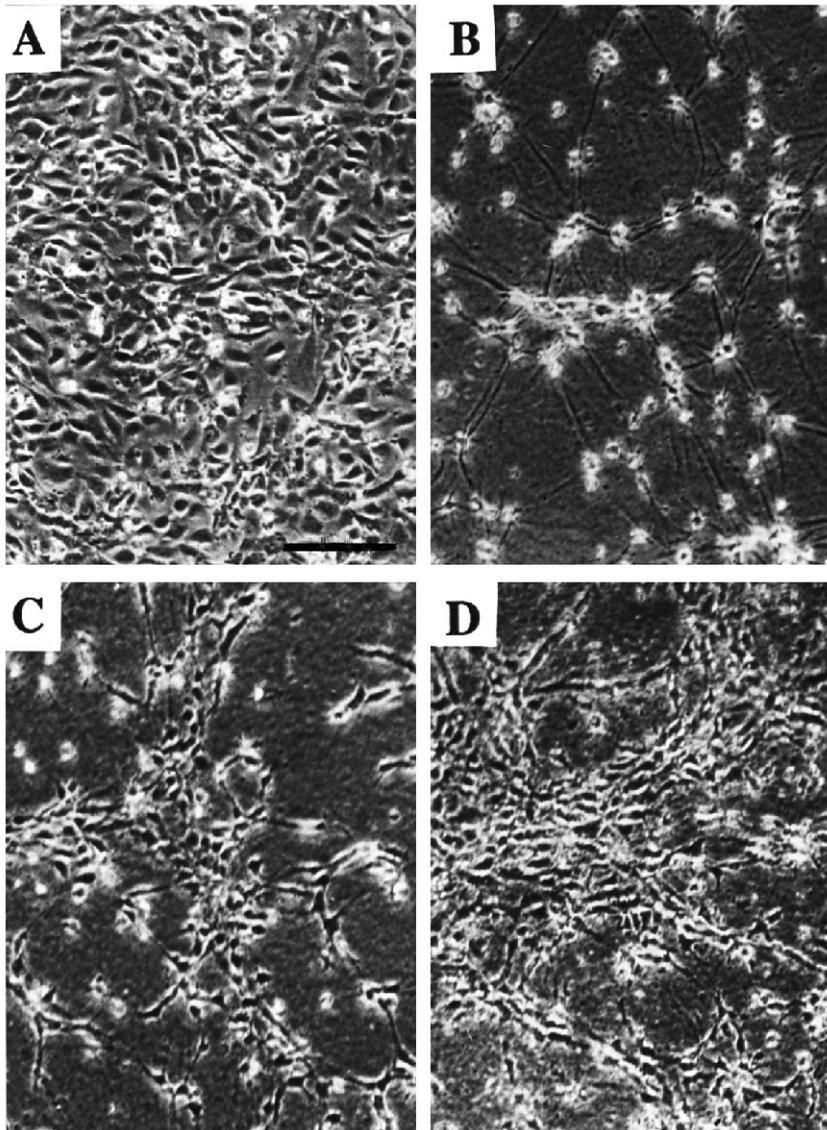
## RESULTS

### Glycosaminoglycans modulate collagen gel contraction by endothelial cells and fibroblasts

Normal human umbilical vein endothelial cells (HUVEC) and human fibroblasts were embedded within a three-dimensional extracellular matrix consisting mainly of collagens I and III. In this environment cells readily formed contact with the polymerized gel extending small dendritic processes visible in phase contrast microscopy. Fibroblasts contracted the gel within hours until a maximum was reached after 24 hours resulting in a cell rich condensed lattice. Also, HUVEC started to contract the gels, however, at a much lower rate than fibroblasts (Fig. 1). After 24 hours the gel surface was reduced to 50% of its original size and slowly continued to contract during an additional 24 hours to 30% of the starting value. In collagen gels evenly dispersed HUVEC gradually arranged into



**Fig. 1.** (A) Comparison of gel contraction by fibroblasts (left) and endothelial cells (right). Identical cell numbers were embedded within the collagen gel and the gel was left to contract after mobilization from the dish. (B) The corresponding surface area was measured after 24 hours of contraction of collagen gels containing either fibroblasts (left) or HUVEC (right).



**Fig. 2.** Cell morphology of HUVEC in monolayer cultures (A) and at different intervals when embedded within three-dimensional collagen gels at 2 hours (B), at 6 hours (C) and 24 hours (D). Bar, 50  $\mu\text{m}$ .

reticular patterns resembling tube-like structures (Fig. 2B-D). This was noted as soon as 6 hours after gel polymerization and was most obvious in contracted gels between 12 and 24 hours.

To study the effects of purified glycosaminoglycans (GAG) on cell-matrix interactions we then added purified GAGs at a final concentration of 0.2% (collagen dry weight/dry weight GAGs) to the solubilized collagen. Gel contraction was monitored for up to 48 hours (data not shown). Influences of glycosaminoglycans on collagen fibrillogenesis were excluded by electronmicroscopic evaluation of the fibrillar pattern and SDS gel electrophoresis revealed no qualitative and quantitative differences (data not shown). Compared with controls (collagen gels without GAGs) there was no major influence on fibroblast gel contraction by the different GAGs (Fig. 3). Hyaluronic acid minimally enhanced gel contraction whereas chondroitin sulfate and dermatan sulfate had no influence at all. Heparin and heparan sulfate were observed to have slightly inhibitory effects on the rate of gel contraction.

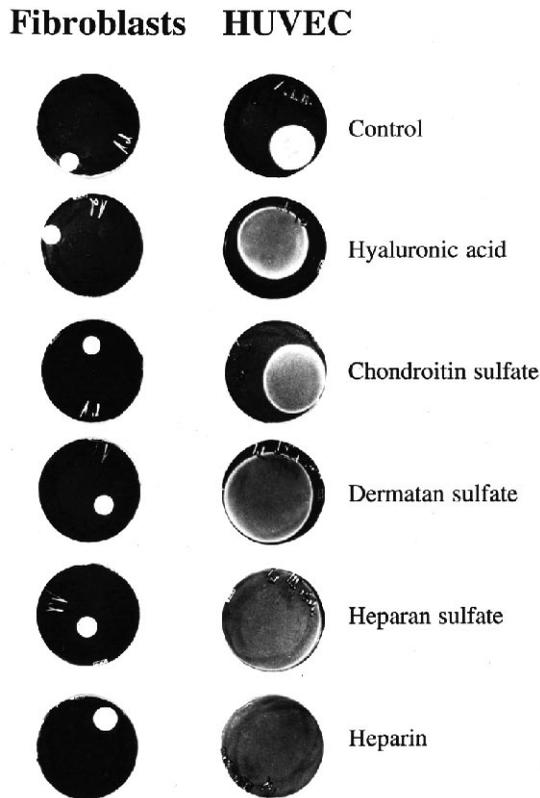
On the other hand, gel contraction by HUVEC was readily modulated by the different GAGs (Fig. 3). Whereas only slight influences were noted after the addition of hyaluronic acid,

chondroitin sulfate and dermatan sulfate, heparan sulfate and heparin inhibited gel contraction almost completely. In addition, as judged from phase contrast microscopy with heparan sulfate or heparin the extent of reticular network formation by HUVEC was less pronounced compared with controls (HUVEC in plain collagen I/III gels, data not shown). The inhibitory effect of heparin on gel contraction was readily reversed by preincubation of heparin with heparitinase. Gels containing digested heparin break down products were contracted to values obtained with plain collagen gels.

To exclude cellular necrosis of embedded cells due to toxic effects of the GAGs or contaminants as a possible cause for non-contraction we determined lactate dehydrogenase [EC 1.1.1.27] activity release into the medium after 24 hours. No correlation of LDH activity and inhibition of gel contraction was observed. Instead, released LDH enzyme activity was close to control values (data not shown).

#### **Metabolic phenotype of HUVEC and fibroblasts in a three-dimensional collagen matrix**

Apart from collagen gel contraction we were interested to

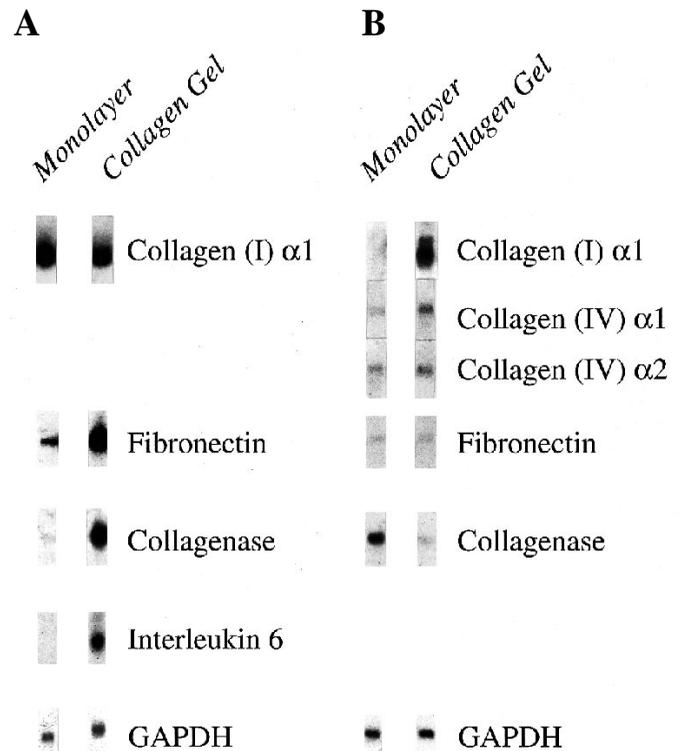


**Fig. 3.** Modulation of HUVEC and fibroblast gel contraction by individual GAGs. Compared with plain collagen I/III gels (controls) different glycosaminoglycans were added to the gels. After 24 hours the contracted gels were photographed. For HUVEC all analyzed GAGs had an inhibitory effect on gel contraction best seen in the case of heparin and heparan sulfate where no contraction was observed.

study the consequences of the different GAGs added to the collagen gels on the metabolic state of both cell types. Since little is known about how a three-dimensional matrix contact regulates steady state mRNA levels we first compared monolayer cultures with fibroblasts and HUVEC embedded in collagen gels. After 24 hours total RNA was prepared and hybridized to specific cDNA probes. We investigated mRNA steady-state levels for extracellular matrix proteins such as collagen I and IV, as well as fibronectin, interstitial collagenase indicating tissue remodelling (synthesis and break down) as well as interleukin-6, an inflammation associated cytokine. These markers should elucidate extracellular matrix metabolism and inflammatory activity of mesenchymal cell types. GAPDH mRNA served as an internal control.

Whereas time course experiments revealed a marked down regulation for collagen I mRNA in fibroblasts within the gel compared with monolayer cultures at 48 hours (data not shown) at 24 hours collagen I levels were only slightly reduced (Fig. 4A). Fibronectin message was substantially induced in fibroblasts within the collagen matrix compared with monolayer culture mRNA levels. Collagenase and interleukin-6 mRNA transcripts were absent in monolayer cultures but grossly stimulated by three-dimensional contact to collagen I/III.

As expected, HUVEC displayed a different mRNA expression pattern from fibroblasts (Fig. 4B). Interestingly, in



**Fig. 4.** (A) Comparison of mRNA steady state levels in fibroblast monolayer cultures and cells maintained in collagen I/III gels. RNA from cells under both culture conditions was isolated after 24 hours culture and hybridized to specific cDNA probes indicated at the right margin. (B) Metabolic consequences of three-dimensional collagen I/III contact in HUVEC. Again, total RNA from monolayer cultures was compared with that of cells embedded within collagen I/III gels for 24 hours. Hybridization probes are indicated.

monolayer cultures little or no collagen  $\alpha 1(I)$  chain mRNA species were noticed. However, in dermal equivalents collagen type I mRNA was significantly induced. Collagen  $\alpha 1(IV)$  and  $\alpha 2(IV)$  mRNAs were present in monolayers and were elevated in collagen embedded HUVEC. Fibronectin mRNA expression was almost undetectable in HUVEC. Neither monolayer culture conditions nor surrounding extracellular matrix affected the expression levels. Collagenase mRNA levels were highly expressed in monolayer cultures but down-regulated to very low levels in dermal equivalents. Interleukin-6 mRNA was not expressed by HUVEC either in monolayer cultures or in cells embedded in collagen gels.

#### Addition of glycosaminoglycans to collagen I/III gels modulate the cellular phenotype

We now wanted to elucidate whether different GAGs, which were shown to modulate gel contraction, also influence the reprogramming of the cellular metabolism due to the contact to three-dimensional collagen matrices.

In fibroblasts GAGs did not modulate collagen  $\alpha 1(I)$  chain specific mRNA levels (Fig. 5A). Fibronectin mRNA levels were slightly down regulated by hyaluronic acid and chondroitin sulfate. This effect was much more pronounced with the addition of dermatan sulfate, heparan sulfate and heparin. Collagenase mRNA expression was slightly inhibited by all incor-

porated GAGs, yet the effect was less obvious than regulation of fibronectin. The strong interleukin-6 mRNA induction by a surrounding collagen matrix was not affected by hyaluronic acid but almost completely diminished by all other added GAGs (with chondroitin sulfate being the least effective one).

Also, HUVEC responded to added GAGs in the collagen lattices with regulation of mRNA species in a cell type specific manner (Fig. 5B). Whereas collagen  $\alpha 1(I)$  mRNA expression was unaffected, collagen  $\alpha 1$  and  $\alpha 2(IV)$  mRNA transcripts were reduced by heparan sulfate and heparin to levels observed in monolayer culture (Fig. 4B). Chondroitin sulfate and hyaluronic acid caused a slight induction of fibronectin mRNA as well as a re-expression of collagenase mRNA absent in plain collagen gels. Interleukin-6 mRNA was not detectable in endothelial cells under these circumstances.

**Glycosaminoglycans regulate mRNA species in endothelial cell and fibroblast monolayer cultures**

GAGs are known to interact with other extracellular matrix proteins influencing the macromolecular organization and also to regulate collagen fibrillogenesis. In order to analyze if the

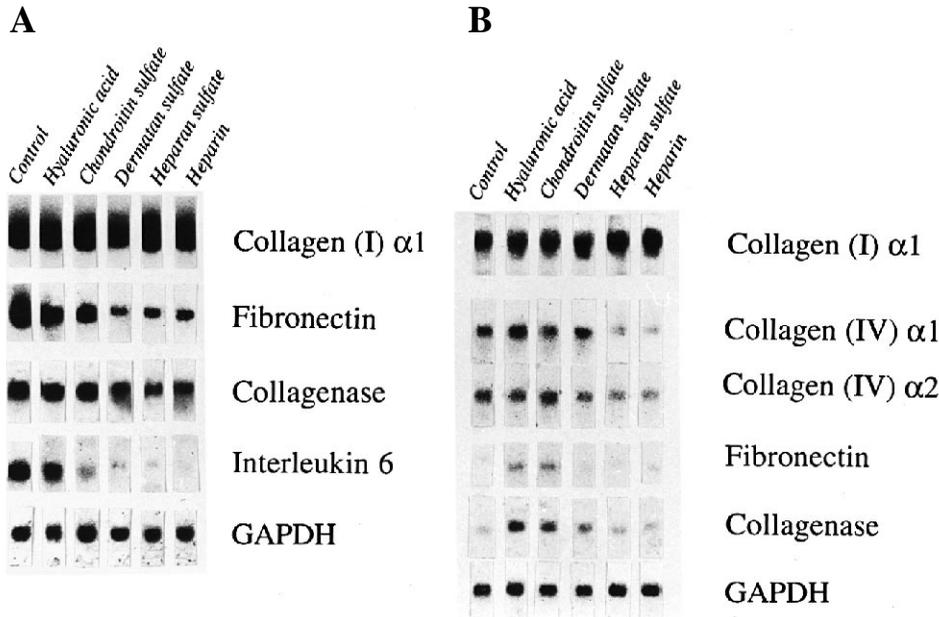
observed alterations in biosynthetic phenotype could be due to a modulation of collagen fibril formation by the GAGs rather than direct GAG-cell contact we also investigated the effect of GAGs on normal monolayer cultures. For this purpose normal monolayer cultures were incubated with different GAGs in the culture medium. Total RNA was extracted after 24 hours.

Neither of the added GAGs did modulate steady-state mRNA levels for collagen I in fibroblasts. In contrast, dermatan sulfate, heparan sulfate and heparin caused an induction of collagenase mRNA (Fig. 6A). Interleukin-6 mRNA levels were not affected (data not shown).

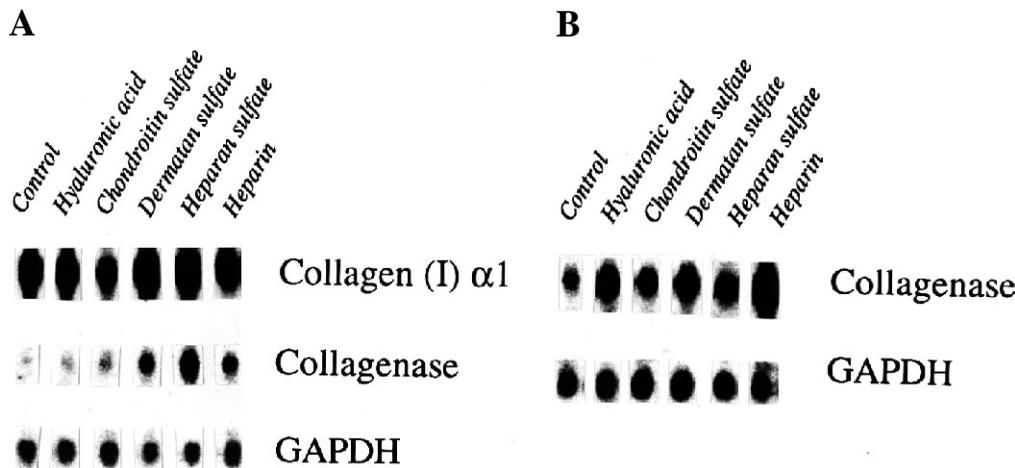
Collagenase mRNA expression in endothelial cells was stimulated by added GAGs, with hyaluronic acid and heparin being most effective, followed by chondroitin sulfate dermatan sulfate and heparan sulfate (Fig. 6B). Again collagen I and interleukin-6 transcripts could not be detected (data not shown).

**Identification of high affinity binding sites for heparan sulfate and heparin on endothelial cells and fibroblasts**

Heparan sulfate and heparin were the most effective GAGs for



**Fig. 5.** (A) Glycosaminoglycans modulate mRNA levels in fibroblasts embedded in collagen gels. Cells in plain collagen I/III gels (controls) were compared with fibroblasts that were maintained in gels containing different glycosaminoglycans. Again RNA samples were hybridized to the indicated cDNA probes. (B) Influence of added glycosaminoglycans on HUVEC embedded in gels for 24 hours. Again, RNA was extracted after 24 hours of culture. The control represents a plain collagen I/III gel. The hybridization probes are indicated.

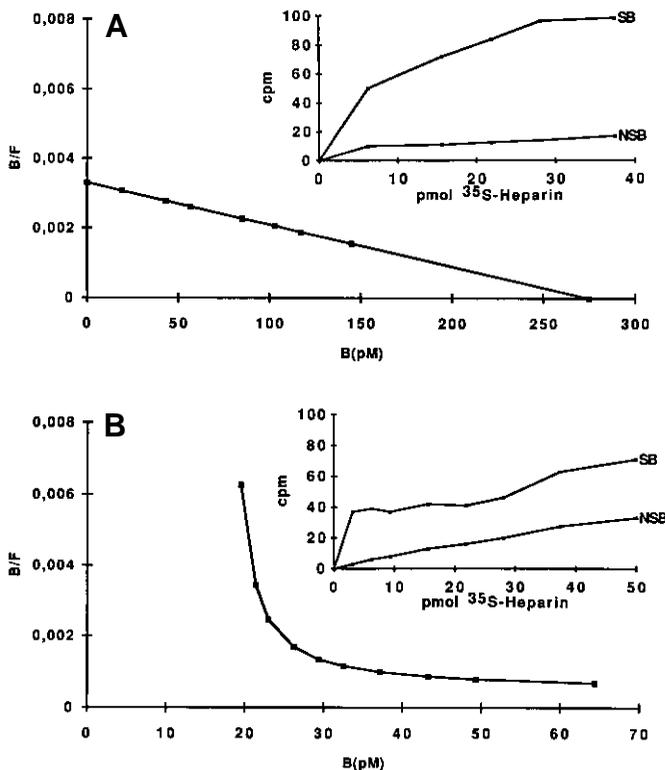


**Fig. 6.** (A) Fibroblasts respond to different glycosaminoglycans in monolayer cultures. Glycosaminoglycans were added to the culture medium (10  $\mu\text{g/ml}$ ) and incubated for 24 hours. Medium was changed in controls without added glycosaminoglycans. (B) Glycosaminoglycans stimulate collagenase mRNA in HUVEC monolayer cultures. Glycosaminoglycans were added to the culture medium as above and incubated for 24 hours.

modulating mRNA levels in fibroblasts and HUVEC when incorporated into dermal equivalents. In addition these GAGs possessed biological activities in monolayer cultures. This led to the question of whether specific binding sites for heparan sulfate and heparin could be identified on both cell types, fibroblasts and endothelial cells. Monolayer cultures were incubated with increasing amounts of  $^{35}\text{S}$ -labelled heparin. Unspecific binding was determined by a 500-fold excess of unlabelled heparin. The resulting binding data were processed according to the method of Scatchard to reveal affinity and number of binding sites per cell.

Fibroblasts displayed a saturable binding affinity for heparin which resulted in a corresponding regression graph (Fig. 7A). The binding affinity was calculated with a  $K_d$  of  $8 \times 10^{-8}$  M and approximately  $2 \times 10^5$  binding sites per cell. Control experiments with mouse 3T3 cells and the anchorage independent U937 cell line revealed binding sites comparable to the above  $K_d$  values and binding characteristics reported previously.

In contrast, HUVEC displayed a biphasic binding behaviour (Fig. 7B). At low heparin concentrations a high proportion of labelled ligand was bound whereas at high ligand concentrations saturation binding was almost obtained. The Scatchard plot revealed two  $K_d$  values: one high affinity binding site with a  $K_d$  value of  $1 \times 10^{-10}$  M and approximately  $5 \times 10^4$  binding



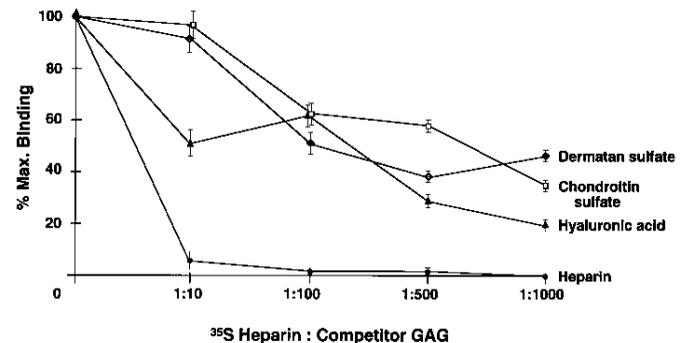
**Fig. 7.** (A) Binding curves for heparin on fibroblast monolayer cultures. Different amounts of  $^{35}\text{S}$ -labelled heparin were incubated (specific binding, SB) or incubated with a 500-fold excess of cold heparin (non specific binding, NSB) with monolayer cultures (insets: each data point is the result of three replicates). Scatchard analysis reveals specific binding sites on fibroblasts. (B) Binding curves for heparin on HUVEC monolayer cultures.  $^{35}\text{S}$ -labelled heparin dilutions were incubated with cells as above (insets). Scatchard analysis reveals a biphasic binding behaviour on HUVEC.

sites/cell and low affinity binding sites with a  $K_d$  of  $4 \times 10^{-6}$  M and  $5 \times 10^5$  binding sites/cell.

Specificity of the binding sites for heparin and heparan sulfate was analyzed by incubating monolayer cultures with 100 ng  $^{35}\text{S}$ -labelled heparin in the presence of different concentrations of unlabelled GAGs in excess. Only heparin was able to effectively block binding at low concentrations of unlabelled GAGs. The amount of bound ligand was determined and is calculated as a percentage of maximal binding (Fig. 8). Further experiments revealed that the binding sites were sensitive to trypsin treatment prior to binding assays, whereas the presence of EDTA (0.1%) had no inhibitory effect (data not shown).

## DISCUSSION

Compared with the *in vivo* situation many cell types display an altered cellular phenotype in tissue culture. An obvious difference to the normal environment is the loss of contact to the surrounding extracellular matrix. Considerable effort has been made to elucidate these cell-matrix interactions on the molecular level. Complex *in vitro* culture systems were developed resembling some aspects of the *in vivo* situation, thus, providing models to study and manipulate cellular functions in an experimental system. Several systems have been characterized in detail. Most information is available for a three-dimensional artificial matrix composed of polymerized collagen which dramatically alters the metabolic state of fibroblasts. In general, cellular proliferation (Nakagawa et al., 1989), differentiation markers (Coulomb et al., 1983), and extracellular matrix protein metabolism are affected (Nusgens et al., 1984; Mauch et al., 1988, 1989) and production of inflammation associated markers is induced (Eckes et al., 1992). Some of these effects are mediated by integrins (Klein et al., 1991; Schiro et al., 1991) expressed on the cell surface (Languino et al., 1989; Kramer et al., 1990; Defilippi et al., 1991). The exact signal transduction pathways are, however, only partly understood (Zachary and Rozengurt, 1992). Embedding endothelial cells in similar experimental conditions in collagen gels resulted in gradual formation of tube-like



**Fig. 8.** Competition of heparin binding on HUVEC by unlabelled GAGs. Binding of 100 ng  $^{35}\text{S}$ -labelled heparin was competed by addition of different concentrations of unlabelled glycosaminoglycan in excess. The resulting competition was compared indicating that heparin and heparan sulfate were most effective at low concentrations whereas even at high concentrations the other GAG could not completely inhibit [ $^{35}\text{S}$ ]heparin binding.

structures, not observed with fibroblasts (Montesano et al., 1983). However, metabolic consequences arising from these endothelial cell-matrix interactions are not well documented.

Most of the experiments used purified collagen type I or mixtures of type I and type III preparations. Only a little information is available for other extracellular matrix components (Kubota et al., 1988; Grant et al., 1989). Proteoglycans can modulate activity of cytokines/growth factors (Ruoslahti and Yamaguchi, 1991) and seem to bind to cells via specific binding sites on a variety of cells and transformed cell lines (Leung et al., 1989; Halper, 1990; Bradbury and Parish, 1991).

When studying the effects of purified extracellular matrix components on cells, inherent limitations include: (1) co-purified matrix bound growth factors affecting the cellular phenotype; and (2) a variable endogenous synthesis of connective tissue components that condition the cellular surroundings. These restrictions are also applicable to the present report, although efforts had been made to exclude unspecific (i.e. not extracellular matrix molecule mediated) effects as far as possible. Heparin or heparan sulfate are both known to have high affinities e.g. for growth factors which could have been co-purified during the GAG isolation procedure. To pursue this possibility further, we digested heparin and observed a reversion of the modulatory effects on the cells back to control values indicating to some extent specific effects of heparin. Conditioning of the close cellular vicinity by endogenously produced matrix components can be monitored and minimized by: (1) analyzing the synthetic phenotype; and (2) choosing short incubation times, thus limiting matrix conditioning. The last aspect necessitated the analysis of cultures after 24 hours, since longer incubation times result in a substantial production of extracellular matrix components and altered cytokine receptor expression (H. Smola et al., unpublished).

Glycosaminoglycans added to gels containing fibroblasts, led to a moderate inhibition of gel contraction (heparin, heparan sulfate) or did not influence the contraction rate at all. This is in contradiction to previous reports, where heparin and heparan sulfate were shown to inhibit gel contraction (Guidry and Grinnell, 1987). However, the authors used isometric conditions and fibroblasts seeded on top of a retained gel. The decrease in gel thickness was measured. As noted above, our system employed isotonic conditions with the surface area being quantitated. Differences in isometric and isotonic cultures conditions were shown previously to be associated with a gross modulation of the cellular phenotype suggesting that both culture systems are not necessarily comparable.

As described before, when fibroblasts were embedded in collagen gels and examined at early time intervals collagen type I mRNA levels did not decrease (Mauch et al., 1988). Yet, in agreement, collagenase mRNA was already induced corresponding to the effects observed here (Mauch et al., 1989). Maintaining fibroblasts in the gels for longer periods resulted in a substantial reduction of collagen I mRNA. However, since fibroblasts condition the surrounding matrix with proteoglycans (Greve et al., 1990) only short incubation times were chosen to investigate GAG effects in order to minimize endogenous conditioning effects. While fibroblasts responded to the different GAGs only slightly in the gel contraction assay, interleukin-6 mRNA levels and collagenase were nevertheless influenced, again suggesting a cell type specific response.

Endothelial cells contracting collagen gels composed of

collagens I/III rapidly formed tube-like structures within 12 hours instead of the reported 2 days (Montesano et al., 1983). These cell matrix interactions were responsible for differences in the synthetic profile. It was interesting to observe that endothelial cells responded to matrix contact in a reciprocal fashion in regard to collagen and collagenase expression compared with fibroblasts, suggesting a different cell type specific response. Three-dimensional collagen I/III contact of endothelial cells seemed to switch these cells from a 'degradative' to a 'synthetic' phenotype.

Using isotonic contraction assays, endothelial cells responded readily to added GAGs. Presence of heparan sulfate or heparin in the gels was accompanied by an almost complete inhibition of the gel contraction and the extent of tube-like networks was largely reduced. Influence of GAGs on collagen polymerization was excluded by electronmicroscopic evaluation of collagen fibrils and protein gel electrophoresis. From this, we conclude that heparan sulfate and heparin were specifically able to reduce gel contraction by HUVEC cells. Lactate dehydrogenase activity levels released into the culture supernatant and the cellular morphology suggested comparable starting numbers of viable cells.

Considering the changes observed in the cellular phenotype it seems reasonable to assume that glycosaminoglycans are involved in modulating steady state mRNA expression levels: (1) in a cell type specific manner; and (2) in that different GAGs selectively modulate cell-matrix interactions. Furthermore, effects of GAGs in monolayer cultures indicate a direct interaction of GAGs and mesenchymal cells.

Modulation of the cellular phenotype by GAGs could be due to several mechanisms. One of these could include receptor mediated binding of GAGs to the cells. We therefore performed binding experiments with radioactively labelled heparin since most of the effects observed were caused by heparin and heparan sulfate. Scatchard analysis revealed specific high affinity binding sites on human dermal fibroblasts and for HUVEC two affinity values were calculated. Competition experiments demonstrated that an excess of unlabelled heparin (as would be expected) and heparan sulfate effectively blocked labelled ligand binding. Yet, inhibition binding experiments with other GAGs implicate that these binding sites do have a certain specificity for the disaccharide composition rather than sulfation grades. This is especially evident when comparing inhibition of heparin binding with hyaluronic acid (no sulfation) and chondroitin sulfate (high sulfation).

Binding of heparin and heparan sulfate via 'bridging' extracellular matrix molecules e.g. fibronectin present at the cell surface of many cell types could explain some of our findings. On the other hand, heparin binding sites have been found on cells usually devoid of a pericellular matrix as in the case of the monocytic cell line U937 or lymphocytes (Leung et al., 1989; Halper, 1990; Bradbury and Parish, 1991). These binding sites were shown to be associated with cellular responses triggered by added heparin (Leung et al., 1989). Furthermore, comparing fibronectin mRNA expression and affinity of binding sites revealed that endothelial cells express almost undetectable amounts of fibronectin mRNA, yet had high affinity binding sites, whereas reciprocal results were obtained with fibroblasts.

These results favour a model for direct binding of glycosaminoglycans or at least heparin/heparan sulfate to fibro-

blasts and HUVEC. At present, however, the exact nature and molecular identity of these binding sites remains elusive. In the biological context, heparan sulfate is an especially interesting molecular modification of the corresponding core proteins. The distribution of heparan sulfate conjugated core proteins showed a pericellular (David et al., 1992) and basement membrane (Iozzo et al., 1994) distribution thus heparan sulfate glycosaminoglycans are in close vicinity of the cells. One could speculate that apart from direct effects on the cells, heparan sulfate could participate in regulation of growth factor activities and half life in the pericellular space.

Our experiments clearly demonstrate for the first time that glycosaminoglycan side chains of proteoglycans can specifically modulate the mesenchymal cell phenotype and even regulate distinct cellular functions. It is now obvious that apart from collagens other extracellular matrix molecules, proteoglycans or glycosaminoglycan side chains as shown here, are able to modulate the mesenchymal cell phenotype. In this context it is obvious that synthesis of these GAGs needs to be closely controlled in processes such as embryonic development or tissue repair during wound healing in order to control cellular functions according to the biological requirements.

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## REFERENCES

- Bell, E., Ivarsson, B. and Merrill, C. (1979). Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. *Proc. Nat. Acad. Sci. USA* **76**, 1274-1278.
- Bernard, M. P., Kolbe, M., Weil, D. and Chu, M. L. (1985). Human cellular fibronectin: comparison of the carboxyl-terminal portion with rat identifies primary structural domains separated by hypervariable regions. *Biochemistry* **24**, 2698-2704.
- Bourdon, M. A., Oldberg, A., Pierschbacher, M. and Ruoslahti, E. (1985). Molecular cloning and sequence analysis of a chondroitin sulfate proteoglycan cDNA. *Proc. Nat. Acad. Sci. USA* **82**, 1321-1325.
- Bradbury, M. G. and Parish, C. R. (1991). Characterization of lymphocyte receptors for glycosaminoglycans. *Immunology* **72**, 231-238.
- Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.
- Chu, M. L., Myers, J. C., Bernard, M. P., Ding, J. F. and Ramirez, F. (1982). Cloning and characterization of five overlapping cDNAs specific for the human pro alpha 1(I) collagen chain. *Nucl. Acids Res.* **10**, 5925-5934.
- Church, G. M. and Gilbert, W. (1984). Genomic sequencing. *Proc. Nat. Acad. Sci. USA* **81**, 1991-1995.
- Coulomb, B., Dubertret, L., Bell, E., Merrill, C., Fosse, M., Breton, G. J., Prost, C. and Touraine, R. (1983). Endogenous peroxidases in normal human dermis: a marker of fibroblast differentiation. *J. Invest. Dermatol.* **81**, 75-78.
- David, G., van der Schueren, B., Marynen, P., Cassiman, J. J. and van den Berghe, H. (1992). Molecular cloning of amphiglycan, a novel integral membrane heparan sulfate proteoglycan expressed by epithelial and fibroblastic cells. *J. Cell Biol.* **118**, 961-969.
- Defilippi, P., van Hinsbergh, V., Bertolotto, A., Rossino, P., Silengo, L. and Tarone, G. (1991). Differential distribution and modulation of expression of alpha 1/beta 1 integrin on human endothelial cells. *J. Cell Biol.* **114**, 855-863.
- Doegge, K., Sasaki, M., Horigan, E., Hassell, J. R. and Yamada, Y. (1987). Complete primary structure of the rat cartilage proteoglycan core protein deduced from cDNA clones. *J. Biol. Chem.* **262**, 17757-17767.
- Eckes, B., Hunzelmann, N., Ziegler-Heitbrock, H. W., Urbanski, A., Luger, T., Krieg, T. and Mauch, C. (1992). Interleukin-6 expression by fibroblasts grown in three-dimensional gel cultures. *FEBS Lett.* **298**, 229-232.
- Feinberg, A. P. and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6-13.
- Gillery, P., Maquart, F. X. and Borel, J. P. (1986). Fibronectin dependence of the contraction of collagen lattices by human skin fibroblasts. *Exp. Cell Res.* **167**, 29-37.
- Grant, D. S., Tashiro, K., Segui, R. B., Yamada, Y., Martin, G. R. and Kleinman, H. K. (1989). Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures in vitro. *Cell* **58**, 933-943.
- Greve, H., Blumberg, P., Schmidt, G., Schlumberger, W., Rauterberg, J. and Kresse, H. (1990). Influence of collagen lattice on the metabolism of small proteoglycan II by cultured fibroblasts. *Biochem. J.* **269**, 149-155.
- Guidry, C. and Grinnell, F. (1987). Heparin modulates the organization of hydrated collagen gels and inhibits gel contraction by fibroblasts. *J. Cell Biol.* **104**, 1097-1103.
- Halper, J. (1990). Specific binding of [<sup>3</sup>H]heparin to human carcinoma SW-13 and other mammalian cells. *Exp. Cell Res.* **187**, 324-327.
- Iozzo, R. V., Cohen, J. R., Grassel, S. and Murdoch, A. D. (1994). The biology of perlecan: the multifaceted heparan sulphate proteoglycan of basement membranes and pericellular matrices. *Biochem. J.* **302**, 625-639.
- Klein, C. E., Dressel, D., Steinmayer, T., Mauch, C., Eckes, B., Krieg, T., Bankert, R. B. and Weber, L. (1991). Integrin alpha 2 beta 1 is upregulated in fibroblasts and highly aggressive melanoma cells in three-dimensional collagen lattices and mediates the reorganization of collagen I fibrils. *J. Cell Biol.* **115**, 1427-1436.
- Kramer, R. H., Cheng, Y. F. and Clyman, R. (1990). Human microvascular endothelial cells use beta 1 and beta 3 integrin receptor complexes to attach to laminin. *J. Cell Biol.* **111**, 1233-1243.
- Krusius, T. and Ruoslahti, E. (1986). Primary structure of an extracellular matrix proteoglycan core protein deduced from cloned cDNA. *Proc. Nat. Acad. Sci. USA* **83**, 7683-7687.
- Kubota, Y., Kleinman, H. K., Martin, G. R. and Lawley, T. J. (1988). Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J. Cell Biol.* **107**, 1589-1598.
- Languino, L. R., Gehlsen, K. R., Wayner, E., Carter, W. G., Engvall, E. and Ruoslahti, E. (1989). Endothelial cells use alpha 2 beta 1 integrin as a laminin receptor. *J. Cell Biol.* **109**, 2455-2462.
- Leung, L., Saigo, K. and Grant, D. (1989). Heparin binds to human monocytes and modulates their procoagulant activities and secretory phenotypes. Effects of histidine-rich glycoprotein. *Blood* **73**, 177-184.
- Marynen, P., Zhang, J., Cassiman, J. J., van den Berghe, D. and David, G. (1989). Partial primary structure of the 48- and 90-kilodalton core proteins of cell surface-associated heparan sulfate proteoglycans of lung fibroblasts. Prediction of an integral membrane domain and evidence for multiple distinct core proteins at the cell surface of human lung fibroblasts. *J. Biol. Chem.* **264**, 7017-7024.
- Mauch, C., Hatamochi, A., Scharffetter, K. and Krieg, T. (1988). Regulation of collagen synthesis in fibroblasts within a three-dimensional collagen gel. *Exp. Cell Res.* **178**, 493-503.
- Mauch, C., Adelman-Grill, B., Hatamochi, A. and Krieg, T. (1989). Collagenase gene expression in fibroblasts is regulated by a three-dimensional contact with collagen. *FEBS Lett.* **250**, 301-305.
- Montesano, R., Orci, L. and Vassalli, P. (1983). In vitro rapid organization of endothelial cells into capillary-like networks is promoted by collagen matrices. *J. Cell Biol.* **97**, 1648-1652.
- Munson, P. J. and Rodbard, D. (1980). Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**, 220-239.
- Nakagawa, S., Pawelek, P. and Grinnell, F. (1989). Long-term culture of fibroblasts in contracted collagen gels: effects on cell growth and biosynthetic activity. *J. Invest. Dermatol.* **93**, 792-798.
- Nusgens, B., Merrill, C., Lapiere, C. and Bell, E. (1984). Collagen

- biosynthesis by cells in a tissue equivalent matrix in vitro. *Coll. Relat. Res.* **4**, 351-363.
- Oldberg, A., Hayman, E. G. and Ruoslahti, E.** (1981). Isolation of a chondroitin sulfate proteoglycan from a rat yolk sac tumor and immunochemical demonstration of its cell surface localization. *J. Biol. Chem.* **256**, 10847-10852.
- Ruoslahti, E.** (1988). Structure and biology of proteoglycans. *Annu. Rev. Cell Biol.* **4**, 229-255.
- Ruoslahti, E. and Yamaguchi, Y.** (1991). Proteoglycans as modulators of growth factor activities. *Cell* **64**, 867-869.
- Saunders, S., Jalkanen, M., O'Farrell, S. and Bernfield, M.** (1989). Molecular cloning of syndecan, an integral membrane proteoglycan. *J. Cell Biol.* **108**, 1547-1556.
- Schiro, J. A., Chan, B. M., Roswit, W. T., Kassner, P. D., Pentland, A. P., Hemler, M. E., Eisen, A. Z. and Kupper, T. S.** (1991). Integrin alpha 2 beta 1 (VLA-2) mediates reorganization and contraction of collagen matrices by human cells. *Cell* **67**, 403-410.
- Schwarz, U., Schuppan, D., Oberbaumer, I., Glanville, R. W., Deutzmann, R., Timpl, R. and Kühn, K.** (1986). Structure of mouse type IV collagen. Amino-acid sequence of the C-terminal 511-residue-long triple-helical segment of the alpha 2(IV) chain and its comparison with the alpha 1(IV) chain. *Eur. J. Biochem.* **157**, 49-56.
- Zachary, I. and Rozengurt, E.** (1992). Focal adhesion kinase (p125FAK): a point of convergence in the action of neuropeptides, integrins, and oncogenes. *Cell* **71**, 891-894.
- Zimmermann, D. R. and Ruoslahti, E.** (1989). Multiple domains of the large fibroblast proteoglycan, versican. *EMBO J.* **8**, 2975-2981.
- Zimmermann, D. R., Dours-Zimmermann, M. T., Schubert, M. and Bruckner-Tuderman, L.** (1994). Versican is expressed in the proliferating zone in the epidermis and in association with the elastic network of the dermis. *J. Cell Biol.* **124**, 817-825.

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