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Transforming growth factors (TGFα and TGFβ1) stimulate chondroitin sulfate and hyaluronate synthesis in cultured rat liver fat storing cells

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The synthesis of total sulfated glycosaminoglycans (GAG) was stimulated by transforming growth factors (TGFα 1.4-fold at 5 ng/ml, and TGFβ1 2.05-fold at 2.5 ng/ml) in primary cultures of rat liver fat storing cells (FSC). The combination of both TGFs resulted in an additively stimulated synthesis of total sulfated GAG (more than 3-fold), chondroitin sulfate (more than 15-fold) and hyaluronate (3.8-fold), respectively, whereas the formation of dermatan sulfate was unchanged and that of heparan sulfate was slightly reduced. In summary, TGFs were identified as important mediators of stimulated GAG synthesis in those cells of the liver (FSC), which are the primary site of matrix glycoconjugate production.

Fat storing cell; Fibrogenesis; Glycosaminoglycans; Chondroitin sulfate; Hyaluronic acid; Transforming growth factor

1. INTRODUCTION

Fat storing cells (FSC), also termed ITO cells, play a central role in liver fibrogenesis [1–5] since under conditions of tissue injury FSC proliferate strongly [6,7] and transform into myofibroblast-like cells [8] producing significant quantities of connective tissue components and, interestingly, a pattern of glycosaminoglycans (GAG) and collagen similar to that found in the fibrotic liver extracellular matrix [1–5]. Studies from others and our laboratory have shown recently that secretions of activated Kupffer cells, monocytes and platelets stimulate proliferation/transformation of FSC in culture [2,7,9] and also the synthesis of proteoglycans [2–4] and hyaluronic acid (HA) [3,10] by these cells. Until now, little is known about the nature of the factors (fibrogenic mediators) stimulating FSC. As we could demonstrate recently, transforming growth factor α (TGFα) stimulates FSC proliferation in a dose-dependent manner [10]. This effect was inhibited strongly by TGFβ [10].

TGFβ regulates cell growth, differentiation and extracellular matrix synthesis in a number of cell types (for review see [12]). Preliminary results that (i) TGFβ stimulates collagen synthesis in FSC, (ii) in acute CCl_4-induced liver damage the level of TGFβ mRNA rises [13] and, furthermore, (iii) TGFβ gene expression is significantly enhanced during active fibrogenesis associated with liver disease in man [14] indicate a central role of TGFβ in hepatic fibrogenesis. The purpose of the present study was to study the stimulatory potency of TGFβ and of the mitogen TGFα on FSC extracellular sulfated GAG and hyaluronate synthesis. Our results indicate that TGFs are potent stimulators of an enhanced chondroitin sulfate and hyaluronate synthesis and secretion by FSC.

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Abbreviations: FSC, fat storing cells; TGFα, transforming growth factor α; TGFβ, transforming growth factor β1; DMEM, Dulbecco's modified Eagles medium; PG, proteoglycans; GAG, glycosaminoglycans; CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; HA, hyaluronic acid

2. MATERIALS AND METHODS

2.1. *Materials*

Collagenase H (clostridiopeptidase A, EC 3.4.24.3), trypsin (EC 3.4.21.4), papain (EC 3.4.22.2) and fetal calf serum were from Boehringer Mannheim, FRG. TGFβ (source human platelets) and TGFα (rat recombinant) were purchased from ICN Biochemicals, Cleveland, OH, USA. [^{35}S]Sulfate (18.5–22.2 GBq/mmol) was from New England Nuclear Corp., Boston, MA, USA, D-[6-^{3}H]glucosamine hydrochloride (1.48 TBq/mmol) was from Amersham Buchler GmbH, Braunschweig, FRG, and Nycodenz (analytical grade) was from Nyegaard and Co., Oslo, Norway. Calf thymus DNA (type I) and pronase were from Sigma Chemical Co., Munich, FRG. Chondroitin AC- (EC 4.2.2.5) and -ABC-lyases (EC 4.2.2.4) were obtained from Seikagaku Fine Chemicals, Tokyo, Japan. The hyaluronate radioligand assay was obtained from Pharmacia, Uppsala, Sweden.

2.2. *Methods*

2.2.1. Cell isolation and cell culture

Detailed procedures of the isolation and culture of FSC from rats have been published previously from this laboratory [2–4,7]. Briefly

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FSC were isolated by a single-step Nycodenz density gradient (8.2%, w/v), seeded with a density of 0.4×10^6 cells/10 cm^2 on 6-well culture plates (Greiner, Nürtingen, FRG) and grown in DMEM (Flow Laboratories GmbH, Bonn, FRG) with 4 mmol/l L-glutamine, 100 kU/l penicillin, 100 mg/l streptomycin and 10% (v/v) fetal calf serum in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The purity of the FSC preparations was assessed by light-microscopy using the presence of perinuclear lipid droplets, the typical cell shape as a marker and by vitamin A-specific autofluorescence. The mean purity of freshly isolated cells was more than 85%, cell viability assessed by trypan blue exclusion was more than 80%. With the first (about 8 h after seeding) and second (about 20 h after seeding) medium change most of contaminating cells were removed, and the FSC monolayers were essentially free of impurities. With the third medium change (about 44 h after seeding) fetal calf serum in DMEM was reduced to 0.5% and test substances (TGFα 0.5–50 ng/ml, TGFβ (0.025–6 ng/ml) were added. Twenty-four hours later growth factors were added again during a further medium change whereby cultures were labeled with radionuclide.

2.2.2. Determination of cell proliferation

DNA measurement: DNA content of the FSC cultures was measured fluorometrically using calf thymus DNA as a standard [15].

2.2.3. Determination of the synthesis of total medium proteoglycans

The synthesis of sulfated GAG was determined by the incorporation of [^{35}S]sulfate (18.5–22.2 GBq/mmol; 740 kBq/ml medium) during a labeling period of 24 h (details described elsewhere [3,4]). Proteoglycans were isolated after solubilization in 7 M urea by binding to DEAE-Sephacel (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated with 0.3 mol/l sodium acetate buffer (pH 6.6), extensively washed with the same buffer, and thereafter eluted using 2.2 mol/l NaCl. The radioactivity of an aliquot of the eluate was counted by liquid scintillation. Proteoglycan synthesis was expressed as radioactivity on the basis of cellular DNA content.

2.2.4. Determination of specific types of sulfated GAG

For analysis of specific types of sulfated GAG the DEAE-Sephacel eluate was subjected to consecutive degradation with nitrous acid to yield the incorporation of label into heparan sulfate (HS) [16] and to enzymatic digestion with chondroitin AC- and -ABC-lyases to obtain the fractions of chondroitin 4,6-sulfate (CS) and dermatan sulfate (DS) [17], respectively. Analytical details of the method are reported elsewhere [18].

2.2.5. Determination of hyaluronic acid synthesis

HA was quantitated according to the method originally described by Laurent et al. [19] in a radioligand assay using a specific HA-binding protein from bovine cartilage.

2.2.6. Determination of GAG transport from intracellular to extracellular space

In a pulse-chase experiment D-[6-^{3}H]glucosamine-labelled GAG were measured in the intracellular space, in the cell surface bound fraction and in medium. Control and TGF-treated cells were labelled for 3 h with D-[6-^{3}H]glucosamine (2600 kBq/culture); thereafter free label was removed and the monolayer was washed 3 × with DMEM. After 0.5, 2.5, 7, 14 and 24 h cultures were stopped by aspirating the medium (medium fraction) and detaching the cells by mild trypsinization (0.05% (w/v) trypsin, 0.02% (w/v) EDTA) for 5–6 min. After centrifugation (10 min, 50 × g) FSC were in the pellet and the cell surface bound fraction in the supernatant. Cells were lysed by repeated freezing and thawing. Labelled GAG of the intracellular space, the cell surface bound fraction and the medium were determined after proteolysis with papain (EC 3.4.22.2) as described previously [4].

3. RESULTS

3.1. *Effect of TGFs on FSC proliferation*

Since FSC transform within one week in culture to myofibroblast-like cells, the experiments described in this report were performed with FSC in primary culture within the first days after seeding. Under the described conditions monolayers were subconfluent at days 3 and 4 after seeding but not yet transformed (as assessed by actin staining). FSC growth measured by DNA content was stimulated slightly by 5 ng/ml TGFα (1.25-fold of control) but inhibited in a dose-dependent manner by TGFβ (0.72-fold of control at 2.5 ng/ml TGFβ). If both factors (20 ng/ml TGFα and 0.5 ng/ml TGFβ) were added together for 48 h DNA content was not significantly different from control.

3.2. *TGF stimulated GAG synthesis and transport*

Pulse-chase experiments with [^{3}H]glucosamine showed that 0.5 h after pulse in controls 53% of the labelled GAG were found in the intracellular space whereas the membrane bound fraction was 33% and medium fraction only 14% (fig.1). Within 7 h most of the intracellularly produced GAG were detected as membrane bound (39%) and medium GAGs (51%) but after 24 h more than 80% of the labelled GAG were found in the extracellular space whereas only 5% remained inside the cell (fig.1). The combination of 20 ng/ml TGFα and 0.5 ng/ml TGFβ elevated GAG synthesis. In TGF-treated cultures 0.5 h after pulse most (81%) of the newly synthesized GAGs were found intracellularly or membrane bound whereas 24 h later only 25% were found intracellularly or cell surface bound. The $t_{1/2}$ of the newly synthesized intracellular

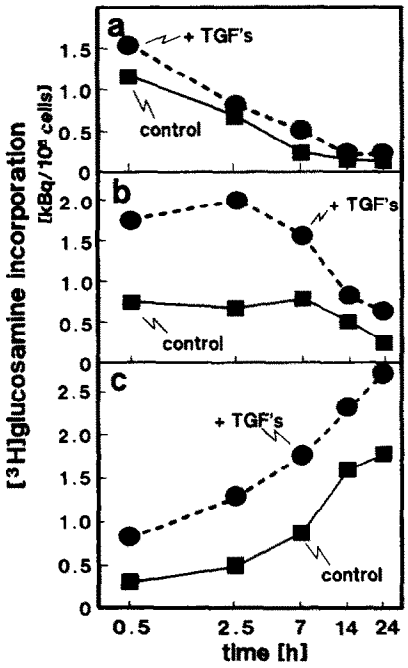

Fig.1. Pulse-chase experiment showing TGF-stimulated GAG synthesis and transport to the extracellular space. D-[6-^{3}H]Glucosamine labelled GAG were measured after proteolysis in the intracellular space (a), in the cell surface bound fraction (b) and in medium (c). The mean values of duplicate experiments are shown.

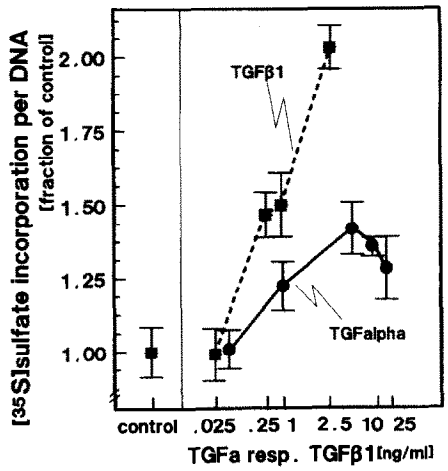

Fig.2. Stimulated incorporation of [^{35}S]sulfate into medium GAG of FSC. TGFs were added in the presence of 0.5% fetal calf serum on the 2nd and 3rd day after seeding. Between the 3rd and 4th culture day cells were exposed for 24 h to 740 kBq [^{35}S]sulfate/ml medium. Values are expressed as mean ± SD of 4 cultures on the basis of DNA.

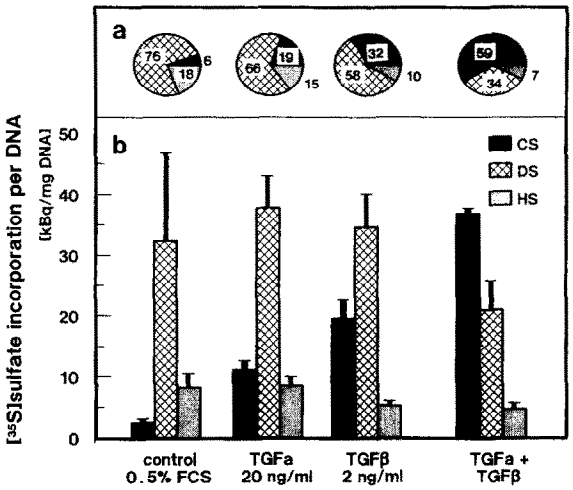

Fig.3. Determination of specific types of sulfated GAG secreted by FSC stimulated by TGFs. TGFs were added alone or in combination (20 ng/ml TGFα and 2 ng/ml TGFβ) on the 2nd and 3rd day after seeding. Between the 3rd and 4th culture day cells were exposed for 24 h to 740 kBq [^{35}S]sulfate/ml medium. (a) The relative amount of specific GAG. (b) Absolute amount of specific GAG. Values are expressed as mean ± SD of 3 cultures, each with $n = 3$ on the basis of DNA. CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate.

GAG decline was similar (about 2.5 h) in controls and TGF-treated cultures. The doubling time of medium GAG was calculated to be 7 h.

3.3. *TGF-stimulated total sulfated medium GAG*

Since, as shown by pulse-chase experiments and previous studies [3,4], more than 75% of the newly synthesized GAG were transported into the extracellular space only medium GAG were measured in further experiments. In the presence of 0.5% fetal calf serum TGFα stimulated medium GAG in a dose-dependent manner (fig.2). Maximum stimulation (1.9-fold per culture and 1.4-fold per DNA) was seen at 5 ng/ml TGFα. TGFβ too caused a dose-dependent enhanced radiosulfate incorporation into secreted GAG with a maximal increase of about 2-fold per DNA at 2.5 ng/ml (fig.2). The combination of TGFα (20 ng/ml) and TGFβ (0.5 ng/ml) elevated the synthesis of total medium sulfated GAG more than 3-fold compared to control.

3.4. *TGF-stimulated chondroitin sulfate synthesis*

In controls (DMEM with 0.5% fetal calf serum) most (76%) of the incorporated [^{35}S]sulfate was found in the fraction of DS whereas HS contained 18% and CS only 6% of the radiosulfate (fig.3a). Addition of TGFβ reduced the relative radiosulfate content of DS from 76% to 58% and HS from 18% to 7%, respectively, but increased the relative [^{35}S]sulfate content of CS from 6% to 32% (fig.3a). The absolute [^{35}S]sulfate incorporation into CS expressed as radiosulfate incorporation per DNA was also significantly stimulated by TGFβ (at 0.5 ng/ml about 7-fold and at 2.0 ng/ml more than 8-fold) compared to control (fig.3b). Similar but quantitatively lower stimulated CS synthesis was seen by adding TGFα to primary cultures of FSC. The combination of TGFα and TGFβ caused a 15-fold enhanced CS synthesis whereas DS and HS synthesis slightly declined (fig.3b).

3.4. *TGF-stimulated hyaluronate synthesis*

TGFα (20 ng/ml) and TGFβ (0.5 ng/ml), respectively, added to FSC cultures stimulated medium HA about 2-fold (fig.4b). The dose-response curve of TGFβ stimulated hyaluronic acid synthesis is shown in fig.4a. In combination TGFs (TGFα 20 ng/ml and TGFβ 0.5 ng/ml) produced an additive stimulation (3.8-fold compared to control) (fig.4b).

4. DISCUSSION

In the present study we could demonstrate for the first time that beside the effects of TGFs on FSC proliferation these growth factors significantly stimulate GAG synthesis and secretion by these cells. Presently the function of the sulfated GAG and other matrix elements secreted by FSC is poorly understood. Several authors propose that the interaction of cells with the extracellular matrix influences cell behavior affecting cell adhesion and motility, growth and differentiation (for review see [20]). Important new aspects of the putative functions of GAG are coming from observations showing that type III TGFβ receptor is a membrane-integrated heparan/chondroitin sulfate proteoglycan [21,22] and that certain GAG act as reservoirs of growth factors [23]. However, up to now we are unable

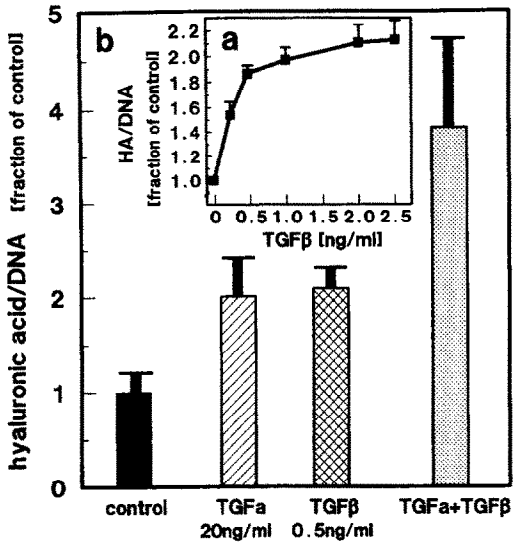

Fig.4. Stimulation of hyaluronic acid synthesis and secretion by TGFα and TGFβ. TGFs were added alone and in combination (20 ng/ml TGFα and 0.5 ng/ml TGFβ) for 48 h between the 2nd and 3rd day after seeding. At day 4 cultures were stopped by aspirating the medium. DNA was measured in the cell layer and HA was estimated in medium by a radioligand assay and expressed on the basis of DNA. (a) Dose-response curve of TGFβ. (b) Effects of TGFs alone and in combination.

to relate the high output of GAG by FSC to specific autocrine or paracrine stimulatory effects.

In cultured normal human dermal fibroblasts TGFβ stimulates the synthesis of types I [24,25] and III [25] collagens and fibronectin [24–26]. In cultured articular chondrocytes [27] and smooth muscle cells [28] TGFβ stimulates GAG biosynthesis. It is suggested, that TGFβ may play a role in normal regulation of extracellular matrix production in vivo and may contribute to the development of skin [25] and organ [29] fibrosis. The results that preferentially the synthesis of CS is stimulated by TGFβ is in agreement with observations made by others in mouse mammary epithelial cells [20] and smooth muscle cells [30]. Further observations that (i) TGFβ is a potent chemoattractant for peripheral blood monocytes [31] and fibroblasts [33], (ii) TGFβ increases cell motility [34], and (iii) TGFβ reduces extracellular protease activity [34] may also be significant in fibrogenesis.

In conclusion, the results obtained lead us to suggest that both TGFα and TGFβ may be candidate mediators of fibrogenic activity in culture but also in vivo during hepatic injury. Their concerted action of FSC might be of significance for the strong accumulation of CS and HA in the extracellular matrix of fibrotic livers.

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