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Non-uniform influence of transforming growth factor-β on the biosynthesis of different forms of small chondroitin sulphate/dermatan sulphate proteoglycan

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The influence of transforming growth factor-β (TGF-β) on the expression of different forms of small proteoglycans was investigated in human skin fibroblasts and in a human osteosarcoma cell line. TGF-β was not found to act as a general stimulator of small proteoglycan biosynthesis. In both cell types, an increased expression of the core protein of proteoglycan I was found. However, there was a profound decrease in the expression of a 106 kDa core protein, and either no alteration or a small decrease in the biosynthesis of the collagen-binding small proteoglycan II core protein. These results show that the production of individual members of the small proteoglycan family is differentially regulated.

INTRODUCTION

Transforming growth factor-β (TGF-β) is known to be a potent regulator of the rate of biosynthesis of several extracellular matrix components, including type I collagen (Roberts *et al.*, 1986; Ignotz & Massagué, 1986; Ignotz *et al.*, 1987), fibronectin (Ignotz & Massagué, 1986), hyaluronan (Westergren-Thorsson *et al.*, 1990) and a variety of chondroitin sulphate/dermatan sulphate proteoglycans (Chen *et al.*, 1987; Bassols & Massagué, 1988; Morales & Roberts, 1988; Rapraeger, 1989). Since TGF-β also up-regulates the production of extracellular matrix receptors (Ignotz & Massagué, 1987; Allen-Hoffman *et al.*, 1988) and stimulates collagen-matrix contraction by fibroblasts (Montesario & Orci, 1988), it has been suggested that this cytokine is a physiological regulator of wound healing.

Small chondroitin sulphate/dermatan sulphate proteoglycan II (PG II; decorin), an abundant extracellular matrix proteoglycan, has been shown to be located at the surface of type I collagen fibrils (Scott, 1988) and to influence collagen fibrillogenesis (Vogel *et al.*, 1984; Hedbom & Heinegård, 1989). Together with PG I (biglycan) it belongs to a family of small chondroitin sulphate/dermatan sulphate proteoglycans which are characterized by core proteins of about 40 kDa (Krusius & Ruoslahti, 1986; Fisher *et al.*, 1989) and the presence of only one (PG II) or up to two (PG I) glycosaminoglycan chains. A recently discovered proteoglycan, PG-100, with a core protein of about 100 kDa (Witsch *et al.*, 1989), is also a small proteoglycan since it elutes from a Sepharose 4B-CL column between PG I and PG II and carries only one or very few chondroitin sulphate chains (K. Schwarz, B. Breuer & H. Kresse, unpublished work). The functions of PG I and PG-100 are not yet known. Since TGF-β acts as a potent stimulator of small proteoglycan biosynthesis in a variety of established cell lines (Bassols & Massagué, 1988), it was tempting to conclude that it is the expression of the ubiquitous PG II which is stimulated by TGF-β (Yamaguchi & Ruoslahti, 1988). It will be shown in this paper, however, that this increase did not occur in two different human cell types and that opposite effects on the secretion of individual proteoglycan types could be observed.

EXPERIMENTAL

Cell culture

Human skin fibroblasts were obtained from a fetus at the 19th week of pregnancy and from juvenile individuals, and were cultivated in modified Eagle's minimum essential medium as described (Glössl *et al.*, 1984). The human osteosarcoma cell line MG-63 was obtained from the American Type Culture Collection (Bethesda, MD, U.S.A.) and cultured under the same conditions. At 2 days before the experiments, cells were trypsin-treated and plated so that the cultures were near confluency after 48 h. Medium was then changed to Waymouth MAB 87/3 medium, which was composed as listed in the Gibco catalogue, except that the glucose concentration was reduced to 7 mM and $MgSO_4$ was replaced by an equimolar amount of $MgCl_2$. The concentration of fetal bovine serum was reduced to 1 %. In some experiments, BSA (10 g/litre; cat. no. 11920; Serva, Heidelberg, Germany) was used in place of serum.

Metabolic labelling

Cultures were preincubated for 24 h with up to 200 pM-TGF-β from human platelets (Paesel & Lorei, Frankfurt, Germany) before the medium was replaced by the same medium which was free of unlabelled leucine and contained 1.5 MBq of L-[4,5-^{3}H]leucine/ml (specific radioactivity 1.8 TBq/mmol; Amersham–Buchler, Braunschweig, Germany) and/or 1.8 MBq of [^{35}S]sulphate/ml (carrier-free; Amersham–Buchler). In some experiments, epidermal growth factor (EGF; Boehringer, Mannheim, Germany) was present in the incubation media along with TGF-β.

Proteoglycan analysis

At the end of the incubation, conditioned media were subjected to an ammonium sulphate precipitation step (Glössl *et al.*, 1984). Precipitated proteins were dissolved in 700 μl of 20 mM-Tris/HCl, pH 7.4, containing 1 M-NaCl, 0.5 % deoxycholate, 0.5 % Triton X-100 and proteinase inhibitors (Glössl *et al.*, 1984) and treated sequentially with Protein A–Sepharose, which had been

Abbreviations used: TGF-β, transforming growth factor-β; PG I, small chondroitin sulphate/dermatan sulphate proteoglycan I (biglycan); PG II, small chondroitin sulphate/dermatan sulphate proteoglycan II (decorin); PG-100, proteoglycan with core protein of ~100 kDa; EGF, epidermal growth factor.

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preadsorbed with an antiserum directed against PG II core protein (Glössl *et al.*, 1984), and PG-100 core protein. Due to the lower titre of the PG-100 antiserum, twice the usual amounts of antiserum and Protein A–Sepharose were used for the preparation of this immune reagent. Details of the purification of PG-100 core protein from the secretions of MG-63 cells and of the immunization procedures will be described elsewhere (K. Schwarz, B. Breuer & H. Kresse, unpublished work). Sequential treatment with either antiserum indicated that the immune precipitations were at least 90% complete. The radioactivity values which were measured in the immune precipitates were used for the calculations without further corrections. The solution remaining after the second immune precipitation step was diluted 6.7-fold with water and chromatographed immediately on a 1 ml DEAE-Trisacryl column as described (Glössl *et al.*, 1984). The proteoglycans which were desorbed with 1 M-NaCl in 20 mM-Tris/HCl, pH 7.4, were brought to dryness and salts were removed by washing with 12.5% trichloroacetic acid followed by washing with methanol. Portions of these preparations and of the immune precipitates were digested with chondroitin ABC lyase (Seikagaku Kogyo, Tokyo, Japan) as described (Glössl *et al.*, 1984) prior to SDS/PAGE (Laemmli, 1970). PG I core protein was quantified by cutting out the appropriate area from the polyacrylamide gel and dissolving the gel in 30% H_2O_2/10 mM-NH_3. Corrections were made according to the recovery of PG II core protein, which was determined by comparing PG II core protein radioactivity before and after SDS/PAGE. It should also be noted that the antiserum directed against PG II did not cross-react with PG I core protein (K. Schwarz, B. Breuer & H. Kresse, unpublished work). The antiserum against PG-100 showed some cross-reactivity with PG II. Immune precipitation was therefore performed as described above. Glycosaminoglycan chains were isolated by a β-elimination reaction and characterized by chromatography on a Sephacryl S-300 column (Greve *et al.*, 1988). Studies on the turnover of secreted PG II were performed as described (Schmidt *et al.*, 1990).

Cell-associated PG II and PG-100 were extracted with 20 mM-Tris/HCl buffer, pH 7.4, containing 1 M-NaCl, proteinase inhibitors and 2% CHAPS for 120 min at 37 °C (500 μl/10 cm^2 growth area). The extract was spun for 20 min at 100000 *g* and the supernatant was treated with preimmune IgG-coated Protein A–Sepharose (Glössl *et al.*, 1984). This treatment was repeated once before PG II and PG-100 were sequentially immune-precipitated.

The influence of TGF-β on collagen biosynthesis was determined according to the conditions described by Roberts *et al.* (1986), except that 1% fetal bovine serum was used instead of plasma-derived serum. Labelling was with 0.37 MBq of L-[U-^{14}C]proline/ml (DuPont de Nemours, Dreieich, Germany) for 24 h, and radioactive collagen was quantitatively measured by degradation with bacterial collagenase (Peterkofsky & Diegelmann, 1971).

RESULTS AND DISCUSSION

Fibroblasts from juvenile donors were preincubated for 19 h with 100 pM-TGF-β and then labelled with [^{35}S]sulphate for an additional period of 4 h. PG II was immune-precipitated from the medium, which contained BSA in place of serum. [^{35}S]Sulphate incorporation into PG II of TGF-β-treated cultures was 104$\pm$7% of that of control cultures ($n = 4$). Since the potency of TGF-β may depend on the simultaneous presence of EGF (Assoian *et al.*, 1983), the effects of applying TGF-β (20 or 200 pM) either alone or in combination with EGF (800 pM) were investigated (Fig. 1). According to the radioactivity which could

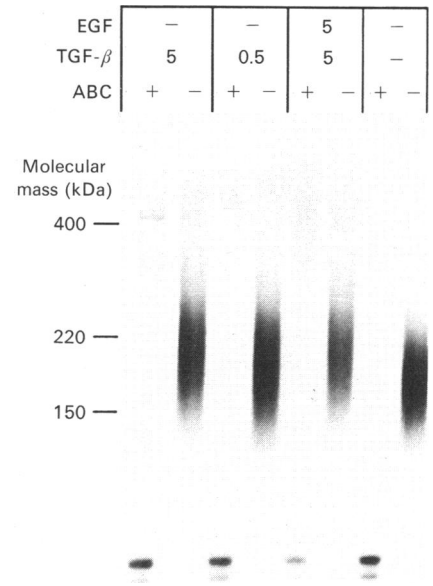

Fig. 1. Influence of TGF-β and EGF on PG II secretion by human fibroblasts

Skin fibroblasts from juvenile donors were incubated for 24 h in Waymouth medium containing BSA in place of serum and the indicated concentrations of growth factors (ng/ml). This pretreatment was followed by a 3 h labelling period with [^{3}H]leucine and [^{35}S]sulphate and a 3 h chase period in the continuous presence of the growth factors. PG II was then immunoprecipitated from the culture medium and subjected to SDS/PAGE in an acrylamide gradient from 3–12.5% after incubation with chondroitin ABC lyase (ABC) or buffer alone. Molecular mass markers (kDa) are indicated.

be immune-precipitated, 200 pM-TGF-β alone caused a stimulation of [^{35}S]sulphate incorporation into PG II by 14%; the combination of both factors, however, led to an inhibition of 8%. Similar changes were seen for ^{3}H labelling. None of these effects were statistically significant. It should be noted that TGF-β was without influence on the size of the dermatan sulphate chain as observed by chromatography on a calibrated Sephacryl S-300 column after β-elimination of PG II (result not shown). This finding is at variance with the effect of TGF-β on syndecan, a cell-surface proteoglycan of epithelial cells, in which an increase in chain length has been found (Rapraeger, 1989). On the other hand, the TGF-β preparation used showed biological activity with respect to collagen biosynthesis. At a concentration of 200 pM, a 2.5-fold stimulation of [^{14}C]proline incorporation into collagen was found (results not shown).

Our failure to observe a significant effect of TGF-β on PG II biosynthesis in fibroblasts from juvenile donors contrasts with the previous finding of an up to 20-fold increase in the expression of various forms of proteoglycans produced in a variety of established cell lines (Bassols & Massagué, 1988). The type of core protein expressed by these cells is not known, whereas secreted proteoglycans from skin fibroblasts cultured in our laboratory contain about 85% PG II, 10% heparan sulphate proteoglycans and 5% large dermatan sulphate proteoglycans (Glössl *et al.*, 1984). PG I and PG-100 are below the limit of detection (Rauch *et al.*, 1986; B. Breuer, G. Schmidt & H. Kresse, unpublished work). To study the effect of TGF-β on the expression of PG I and PG-100, we have therefore used MG-63 osteosarcoma cells and fetal fibroblasts, which synthesize one or both of these proteoglycans in addition to PG II.

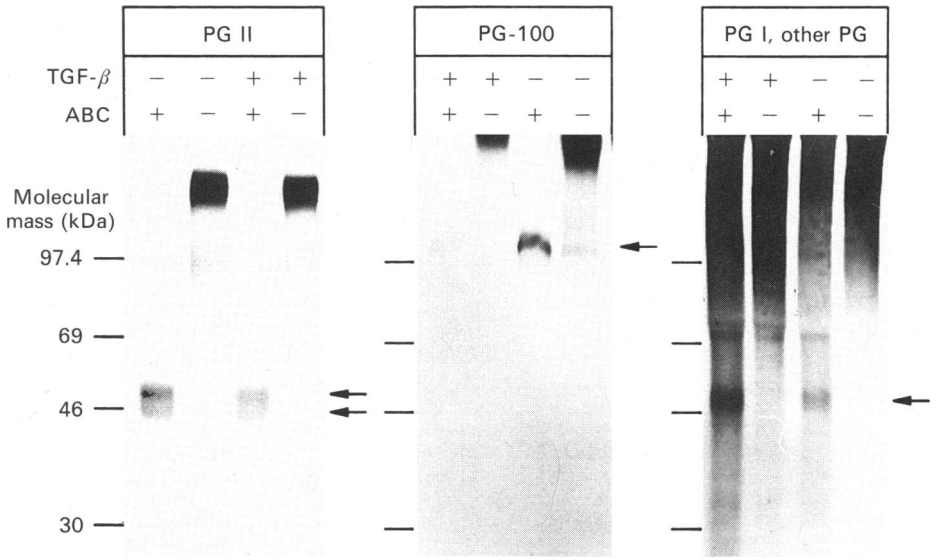

Fig. 2. Influence of TGF-β on small proteoglycan production by the osteosarcoma cell line MG-63

Cells were preincubated for 24 h in Waymouth medium containing 1% fetal calf serum in the presence or absence of 200 pM-TGF-β. All other conditions were as described in the legend of Fig. 1, except that the acrylamide concentration of the separation gel was 12.5%. The arrows indicate the positions of the respective core proteins.

After incubation as described in the legend of Fig. 2, [^{35}S]sulphate incorporation into total proteoglycans was increased by 1.4-fold under the influence of TGF-β in osteosarcoma cells, and by 1.5-fold in fetal fibroblasts. When fetal bovine serum was replaced by BSA, this increase was slightly reduced in fibroblast cultures (135% of untreated control), but unaltered in osteosarcoma cell cultures. Similar data on the enhancement of proteoglycan production were reported by Westergren-Thorsson *et al.* (1990) using human embryonic skin fibroblasts.

Sequential immune precipitation of PG II and PG-100 made possible the specific quantification of these two proteoglycans. PG I core protein, which behaves as a 54 kDa protein during SDS/PAGE, could be quantified only after chondroitin ABC lyase digestion of PG I and electrophoretic separation from heparan sulphate proteoglycans. The data provided in Fig. 2 and Table 1 allow the following conclusions. Firstly, in fetal fibroblasts and osteosarcoma cells there was again no stimulation of the biosynthesis of PG II, but even a slight reduction. Secondly, in three independent experimental series, about a 2-fold increase in the labelling of PG I core protein was found in the presence of TGF-β. Thirdly, treatment with TGF-β led to a diminished incorporation of [^{3}H]leucine and of [^{35}S]sulphate into PG-100. The decreases in the amounts of secreted PG II and PG-100 were not the result of inadequate secretion or increased cell association of these two proteoglycan species. Extracts of the cell layer of MG 63 cells contained, after labelling with [^{35}S]sulphate, 15–24% of secreted PG II and 4–7% of secreted PG-100, regardless of whether the cultures had been treated with TGF-β or not. In fetal fibroblasts, 6–8% of secreted PG II was extracted from the cell layer, and an effect of TGF-β could not be observed. This suggests that the cytokine exhibits a direct influence on the production of these proteoglycans and not on their secretion.

It had been shown previously (Schmidt *et al.*, 1990), by adding affinity-purified antibodies against PG II into the culture medium and thereby inhibiting endocytosis, that PG II can be rapidly internalized and degraded by fibroblasts in monolayer cultures. Applying the same protocol for an investigation of the turnover of PG II in the presence of TGF-β, it could be shown that, in fibroblasts, TGF-β did not exert an influence on the turnover of the proteoglycan. Parallel incubations for 4 h in the presence or absence of 200 pM concentrations of the cytokine differed by up to 8%, and at the highest antibody concentration (5.75 μg/ml) the amount of secreted PG II was 1.6 times greater than in the presence of the same amount of control IgG. As affinity-purified antibodies against PG-100 and PG I are not available, similar investigations on the influence of TGF-β on the metabolism of these proteoglycans could not be performed.

In summary, our results clearly show that the biosynthesis of individual members of the small proteoglycan family is differentially regulated. They do not support the hypothesis that TGF-β is an inducer of PG II biosynthesis. Cheifetz & Massagué (1989) were also unable to detect increased PG II expression in

Table 1. Influence of TGF-β on small proteoglycan core protein expression

Three cultures were each incubated in the absence or presence of 200 pM-TGF-β in Waymouth medium containing [^{3}H]leucine, [^{35}S]sulphate and 1% fetal bovine serum as described in the Experimental section. Mean values ± S.D. are given for ^{3}H incorporation. Values in parentheses indicate % of control incorporation (in the absence of TGF-β). The ^{35}S/^{3}H ratios of the mean values were between 2.3 and 1.9 for PG II and between 1.8 and 1.7 for PG-100. The coefficients of variation of ^{35}S incorporation were up to 26%, and the influence of TGF-β on the ^{35}S/^{3}H ratio was not significant.

		$10^{-5} \times {}^{3}$H incorporation (c.p.m./mg of cell protein)	
Proteoglycan	TGF-β	Fetal skin fibroblasts	Osteosarcoma cells
PG I	–	0.75 ± 0.30 (100)	1.17 ± 0.21 (100)
	+	1.36 ± 0.19 (181)	2.74 ± 0.31 (234)
PG II	–	0.82 ± 0.14 (100)	0.53 ± 0.10 (100)
	+	0.62 ± 0.06 (76)	0.30 ± 0.03 (57)
PG-100	–	Trace	1.80 ± 0.35 (100)
	+	Trace	0.38 ± 0.04 (21)

response to TGF-β in CHO cells. These results do not argue against a potential role of PG II in tissue repair and wound healing. In monolayer cultures of skin fibroblasts, PG II follows a secretion–recapture pathway if the proteoglycan is not bound to an extracellular matrix component (Schmidt *et al.*, 1990). It could be speculated, therefore, that the amounts of PG II which are normally produced are sufficient to cope with an increased demand during repair.

The only proteoglycan which was overexpressed under the influence of TGF-β was PG I. This proteoglycan apparently does not interact with collagen (Hedbom & Heinegård, 1989), and it may be a characteristic matrix component of fetal tissues (Vogel & Evanko, 1987). Thus it seem conceivable that PG I may also be involved in tissue remodelling and in tissue repair.

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