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Transforming Growth Factor β Regulates the Metabolism of Proteoglycans in Bovine Cartilage Organ Cultures*

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The effect of transforming growth factor β (TGF-β) has been studied in a bovine articular cartilage organ culture. The peptide stimulates synthesis of proteoglycans in a dose-dependent manner, reaching saturation at 10 ng/ml. This dose gave an approximate 7-fold increase in synthesis over basal controls. In addition, the peptide decreased the rates of catabolism of proteoglycans with an approximately 2-fold maximal effect seen at 5 ng/ml. At the latter concentration, TGF-β prevented the 4-fold loss of proteoglycans which occurred in cultures maintained under basal conditions over the course of 3 weeks. There was no increase in cell (DNA) content of the cartilage explants under these conditions of TGF-β treatment, and the net collagen content of the explants remained constant.

The resiliency of cartilage results from the swelling pressure created by entrapment of the highly anionic proteoglycans within the fibrillar collagen network (1). This property of cartilage is critical for the protection of underlying bone. Under normal conditions, resident chondrocytes regulate the resiliency of cartilage by controlling the rates of biosynthesis and catabolism of proteoglycans (2). However, during degenerative disease, for example osteoarthritis, the rates of catabolism override deposition of newly synthesized molecules in the matrix. This results in net loss of proteoglycans, a condition that worsens with progression of the disease (3). Thus, knowledge of the nature and interplay of regulatory factors that maintain and/or restore a steady state metabolism of proteoglycans in cartilage is critical to the understanding of pathophysiological processes in the joint and to eventual pharmacological intervention to halt disease.

Transforming growth factor β which exists in two homologous homodimeric forms, TGF-β1[1] and TGF-β2, has been shown to enhance synthesis of extracellular matrix components including collagen, fibronectin, and proteoglycans (for review, see Ref. 4). Furthermore, the studies of Seyedin *et al.* (5) show that TGF-β has the ability to stimulate cultured mesenchymal cells to express the cartilage phenotype as shown by synthesis of proteoglycans and type II collagen. It was postulated that TGF-β may be involved in cell differentiation, including cartilage formation during the first step of endochondral bone formation. In addition to these effects on the biosynthesis of matrix constituents, TGF-β has the ability to decrease the activity of various extracellular proteinases found in connective tissues, including collagenase, stromelysin, and plasminogen activator (4, 6–8).

Such studies on isolated cells have yielded considerable information concerning the mechanism of action of TGF-β, especially at the gene level. For example, it has been shown that TGF-β stimulates the promoter for the type I collagen (9) and increases transcription of the fibronectin gene (10). However, isolated cell cultures have not yielded information on the regulatory mechanisms whereby adult tissues coordinate the processes of biosynthesis and catabolism. We studied the effects of the peptide on a stable articular cartilage organ culture that had previously been characterized with respect to the metabolism of proteoglycans (11). We show that TGF-β (5–10 ng/ml) has the ability to increase rates of biosynthesis of proteoglycans and to depress rates of catabolism. Furthermore, in contrast to basal control cultures which lose proteoglycans from the extracellular matrix TGF-β is able to orchestrate biosynthetic and catabolic pathways to maintain a high concentration of proteoglycans for at least 3 weeks in culture without modulating DNA content. This suggests that TGF-β may play a physiological role in the maintenance of cartilage homeostasis with respect to proteoglycan metabolism.

MATERIALS AND METHODS

General Culture and Labeling Procedures—Articular cartilage was dissected from the metacarpalphalangeal joints of calves of approximately 1–6 months (slaughterhouse estimate) as described before (11, 12). The cartilage slices were thoroughly minced and maintained in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose and 0.58 g/liter L-glutamine (DMEM) in batch cultures for a period of 5–7 days (30 volumes of media to wet weight of tissue, ml/g). When appropriate, measurement of wet weights was obtained by transferring tissue portions (approximately 50 mg) into preweighed Falcon Petri dishes containing 1.5 ml of medium. The weighed tissues or approximately equivalent tissue portions were transferred into the wells of a 24-well Costar tissue culture plate prior to initiation of experimental treatments. Medium was changed daily throughout the culture period.

For both the biosynthetic and catabolic experiments, unless otherwise indicated, the basal controls were defined as the cultures maintained in DMEM media containing 0.1% bovine serum albumin, and the experimental samples were cultured in the same medium containing 5 ng/ml TGF-β. Positive controls consisted of cultures treated with 20% fetal calf serum (FCS), previously shown to maintain steady state metabolism of proteoglycans.

For biosynthetic experiments, the tissue portions were maintained under either of the treatment conditions defined above and on the day indicated, all samples were radiolabeled as follows. First, the conditioned medium from each culture was replaced by 1.5 ml of fresh DMEM, and the tissues were incubated for 30 min at 37 °C to allow replenishment of fresh nutrients to the cells. This medium was then replaced by radiolabeling solution, 1.5 ml of DMEM containing 20 μCi/ml [^{35}S]sulfate for 2–4 h (37 °C). Following incubation, the labeling medium was discarded and the tissues dispersed by treatment with proteinase K (1 mg/ml, 60 °C, 16 h).

For catabolic experiments, separate tissue portions were labeled in a batch under the same conditions as used for the biosynthetic experiments except that labeling was overnight. The labeled tissue was rinsed 3 times with 15 volumes (ml/g) of medium to help remove

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[1] The abbreviations used are: TGF, transforming growth factor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; IGF, insulin-like growth factor.

unincorporated isotope and was then cultured for another 2 days to ensure complete removal (11). Fifty- to one hundred-mg portions of the tissue were transferred to Costar tissue culture wells for the period of chase (18 days). Previous work (2) showed that during the chase the tissue cultured under basal conditions releases partially degraded molecules into the medium, indicative of a proteolytic (catabolic) process. In the present experiments the cartilage tissue was maintained in culture medium containing the appropriate additions during the chase. The daily conditioned medium was stored at −20 °C and analyzed for ^{35}S activity content at the end of the experiment along with the corresponding tissue extract prepared by proteinase K digestion.

The $t_{½}$ was defined as the time required for 50% of the radioactivity present in the tissue on the second day after the pulse to be released into the medium.

TGF-β was purified to homogeneity as described before (13) except that urea was removed by high pressure liquid chromatography on a C18 reverse phase μBondapak column.

Tissue Analysis—Collagen content was determined from hydroxyproline measurements by the procedure of Woessner (14) in hydrolyzed portions of the proteinase K extracts. Glycosaminoglycan content of the extracts was estimated by the dimethylmethylene blue binding procedure (15). Since the predominant glycosaminoglycan in the bovine cartilage is chondroitin sulfate (approximately 90%), standard curves were constructed using this glycosaminoglycan (chondroitin sulfate C from shark cartilage, Seikagaku Kogyo Co., Ltd.). DNA content was determined using bisbenzimidazole (Hoechst 33258) (16). Biosynthesis was routinely measured by quantitation of macromolecular ^{35}S after removal of unincorporated label from the tissue extracts by Pharmacia PD10 chromatography (12). These values were expressed as disintegrations/min of [^{35}S]sulfate incorporated per h since incorporation is linear within the 2–4-h labeling period and normalized to hydroxyproline content since previous work (12) showed that this is constant throughout the culture period.

RESULTS

Bovine articular cartilage cultured in the presence of serum maintains steady state amounts of proteoglycans in the matrix for at least 3 weeks by regulating both biosynthesis and catabolism (11, 12). Collagen is metabolized very slowly in this system but is also maintained at steady state concentration. When serum is removed from the system there is a progressive loss of proteoglycans due to decreased biosynthesis and increased catabolism. Nevertheless, the serum-free cultures maintain phenotypic stability and synthesize proteoglycans and type II collagen characteristic of cartilage.

In the present set of experiments we studied the bovine organ cultures maintained under either of the following conditions: 1) DMEM containing 0.1% bovine serum albumin (basal controls); 2) same as 1, but containing TGF-β; and 3) DMEM containing 20% FCS.

Fig. 1 shows that TGF-β treatment for 6 days increases incorporation of [^{35}S]sulfate into macromolecular components in bovine cartilage organ cultures as compared to basal control cultures. In this system >95% of the [^{35}S]sulfate precursor is incorporated into proteoglycans (11, 12), and thus ^{35}S incorporation can be used directly to assess proteoglycan synthesis. The biosynthetic response to TGF-β treatment is dose-dependent, reaching saturation at 10 ng/ml. The stimulation over control cultures is 5.5-fold for 20% fetal calf serum and 6.6-fold for 10 ng/ml TGF-β in the experiment shown in Fig. 1. In a series of experiments with six different animals, TGF-β at 5 ng/ml stimulates proteoglycan synthesis of basal cultures 3.9 ± 1.6-fold compared to a 3.5 ± 1.3-fold stimulation by FCS (mean stimulation ± S.D.).

In a separate experiment, cultures were maintained under basal control conditions for a week and then maintained for 21 days under the three experimental protocols described above to determine the long-term effects of TGF-β on the regulation of proteoglycan synthesis. A concentration of 5 ng/ml TGF-β was chosen, since this dose gave comparable effects

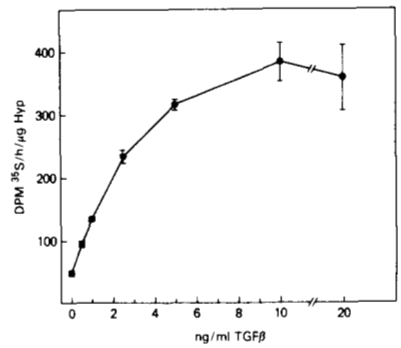

FIG. 1. **Dose response of proteoglycan synthesis to TGF-β.** Tissues were cultured in DMEM + 0.1% bovine serum albumin or this medium was adjusted to the concentration of TGF-β indicated by addition of a 60-fold concentrated TGF-β solution to the culture medium each day (after the medium change). Treatment was for 6 days. Duplicate samples were cultured for each concentration, and the *error bars* indicate the range. *Hyp*, hydroxyproline.

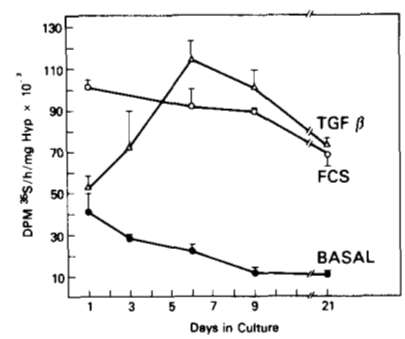

FIG. 2. **Biosynthesis of proteoglycans in long-term cultures.** Cartilage cultures were maintained in a batch in DMEM as indicated under "Materials and Methods," treated under various conditions as indicated in the figure, and pulsed. Each sample was run in duplicate, and the range is indicated. *Hyp*, hydroxyproline.

to 20% FCS in the biosynthetic experiments. Fig. 2 shows that in basal control cultures there is a progressive decrease in the rates of proteoglycan synthesis to 25% of initial rates during the first 9 days of the experiment. The low rates then remained stable between days 9 and 21. Fetal calf serum (20%) increased biosynthetic rates over 3-fold after 24 h and maintained rates within 70% of initial rates for 21 days. In contrast to the full response of these cultures to fetal calf serum within the first 24 h, there was a considerable lag time in the response to TGF-β. The peptide (5 ng/ml) did not elicit a significant response after 24 h, produced a stimulation 60% of maximal after 3 days, and gave maximal stimulation (5.2-fold) only after 6 days of treatment.[2] Following the lag period, stimulation of synthesis by TGF-β was comparable to that of fetal calf serum for the rest of the culture period.

Fig. 3 shows the results of a pulse-chase experiment carried out with another portion from the same batch of tissue used for the biosynthesis experiments shown in Fig. 2. The tissues were pulsed with [^{35}S]sulfate under basal conditions, and the amount of ^{35}S-labeled material remaining in the matrix on each day of the chase was calculated (see "Materials and Methods"). The $t_{½}$ (time required for 50% release of ^{35}S-proteoglycans from the matrix)[3] in the presence of 20% fetal

[2] In another experiment the cultures showed only 20% of the maximal stimulation on day 3 followed by a 6-fold stimulation on day 6.

[3] Previous work has shown that rates of release of ^{35}S-labeled proteoglycans are identical to rates of release of unlabeled proteoglycans.

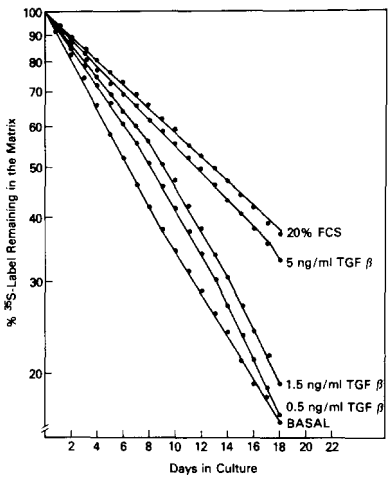

FIG. 3. **Catabolism of proteoglycans.** The cultures were pulsed overnight in the presence of fetal calf serum, washed for 2 days in the same medium (see "Materials and Methods"; data points for these 2 days are not shown in figure), and then duplicate cultures chased for the indicated days under various conditions as indicated in the figure.

TABLE I

Effect of TGF-β on the collagen and DNA content of the tissue

Day of treatment	Sample	Hydroxyproline/ initial wet weight	DNA/ hydroxyproline
		μg/mg	*μg/mg*
Day 0		23 ± 1	69 ± 10
Day 9	Basal control	23 ± 2	
	TGF-β	27 ± 3	
	20% FCS	26 ± 1	
Day 21	Basal control	23 ± 2	53 ± 1
	TGF-β	23 ± 1	46 ± 9
	20% FCS	23 ± 1	61 ± 8

The values represent the average for duplicate cultures ± range.

calf serum was 13.5 days in contrast to the higher rates of release observed in the absence of serum ($t_{½}$ = 6.5 days). There was a dose-dependent decrease in the catabolic rates with TGF-β treatment relative to control cultures ($t_{½}$ = 8, 9, and 12 days for 0.5, 1.5, and 5 ng/ml, respectively). The highest concentration of TGF-β tested (15 ng/ml, not shown) did not further reduce the catabolic rate and in fact gave a lesser effect than 5 ng/ml ($t_{½}$ = 10 days).

The effect of long-term treatment of the cartilage organ cultures with 5 ng/ml TGF-β on the net amount of tissue collagen and glycosaminoglycan was determined for the tissues examined in Fig. 2. The wet weights of all cultures were determined on day 0 when the experimental treatments were initiated, and at the end of the experiment the amount of hydroxyproline (collagen) per initial wet weight was determined. This analysis rules out variability between samples due to any wet weight or dry weight changes during the culture period. The net amount of collagen per initial wet weight is constant in all of the cultures during the culture period (Table I) under the experimental conditions. In contrast to the stability of the collagen component, the proteoglycan (glycosaminoglycan) content of the tissue was progressively depleted in basal controls so that the values on day 21 (460 ± 280 μg/mg hydroxyproline; average ± S.D. for 4 replicate cultures) were 76% lower than those on day 0 (1900 ± 220 μg/mg hydroxyproline, n = 3) (Fig. 4). In this representative experiment, TGF-β prevented chondroitin sulfate loss; the tissues

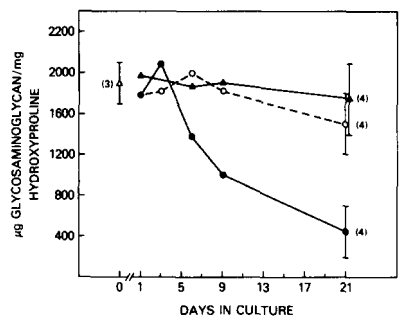

FIG. 4. **Glycosaminoglycan content of the cultured tissues.** The glycosaminoglycan content was determined by the dimethylmethylene blue binding procedure in aliquots of the proteinase K extracts analyzed in Fig. 2. The *numbers* in *parentheses* indicate the number of replicate cultures analyzed, and the *error bars* are the standard deviations for these points. For all time points, cultures were run with at least two replicates. ●——●, basal controls; O- - -O, TGF-β-treated cultures; ▲——▲, FCS-treated cultures.

contained 1490 ± 290 μg/mg hydroxyproline (n = 4) after 21 days in the presence of the factor. Fetal calf serum was also able to maintain a high tissue chondroitin sulfate concentration (1780 ± 390 μg chondroitin sulfate/mg hydroxyproline, n = 4) as demonstrated previously (11).

The net amount of DNA in the tissues at the beginning (day 0) and the end of the experimental treatment (day 21) was determined, and the results are shown in Table I. The increase in biosynthesis of proteoglycans induced by TGF-β (5 ng/ml) or fetal calf serum compared to control cultures was not accompanied by an increase in DNA content, indicating an increased biosynthetic activity/cell.

DISCUSSION

We present evidence that TGF-β has the ability to increase the biosynthesis and decrease the catabolism of proteoglycans in a fully differentiated bovine cartilage matrix cultured in the absence of serum. Furthermore, unpublished biosynthetic experiments[4] have shown that like FCS, TGF-β increases incorporation of [^{35}S]sulfate into proteoglycan aggregate structures, the critical structures involved in maintaining the resiliency of the tissue. The overall effect of the peptide factor is to prevent the loss of proteoglycans that occurs under basal conditions in the cultured tissues.

We used conditions previously defined for the characterization of an organ culture system that is well suited for the study of proteoglycan metabolism. Under these conditions the overall collagen content of the explants remains constant allowing the study of changing proteoglycan parameters within a constant collagen value. It is worth pointing out that the culture conditions are not optimized for collagen synthesis and that the experiments do not rule out the possibility that under appropriate conditions TGF-β may stimulate the synthesis of one or several of the collagen types found in cartilage.

The stimulation of synthesis and depression of catabolism of proteoglycans was comparable to that of FCS. It is likely that the effect of serum on cartilage proteoglycan metabolism is complex and that there are several contributing factors, including mitogens. It has been demonstrated that TGF-β in serum is biologically latent (17), and thus for serum TGF-β to contribute to the effect would require an activation mechanism, perhaps by the chondrocyte. On the other hand, McQuillan *et al.* (18) reported that the effect of FCS on bovine articular cartilage explants could be blocked by antibodies to IGF-1. Recent work indicates that recombinant IGF-1 (20 ng/

[4] T. I. Morales and A. B. Roberts, unpublished observations.

ml) when added to basal control cultures of bovine articular cartilage mimmicks the effects of FCS on biosynthesis and catabolism of proteoglycans (19). It will therefore be critically important to determine whether IGF-1 and/or TGF-β are involved in the autocrine regulation of cartilage homeostasis and how these two factors interact.

mRNA coding for TGF-β has been isolated from cultured chick embryo chondrocytes (20) and rat chondrosarcoma cells (21). Furthermore, immunolocalization studies using antibodies specific for TGF-β have shown intense staining in areas of beginning chondrogenesis during vertebrae formation in 13-day mouse embryos (22) and in chondrocytes within bovine articular cartilage of 6-month-old fetuses (23). It will be interesting to determine if mature articular cartilage retains the ability to synthesize TGF-β and whether the chondrocyte utilizes this factor to modulate proteoglycan metabolism in response to environmental demands.

In summary, TGF-β modulated both the anabolism and catabolism of proteoglycans to maintain tissue homeostasis with respect to proteoglycan concentration in articular cartilage. This supports the hypothesis that TGF-β regulates the overall metabolism of matrix constituents in connective tissues and specifically suggests that TGF-β may be an important pathophysiological regulator of cartilage proteoglycan metabolism.

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