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TGF beta 1 and biglycan, decorin, and fibromodulin metabolism in canine cartilage

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Summary

Objective: Small proteoglycans (PGs) may accumulate in late stage osteoarthritis even as aggrecan is lost. It is not clear what role transforming growth factor (TGF) beta has in this accumulation. Our goal was to investigate the ability of TGF beta 1 to modulate the synthesis and accumulation of decorin, biglycan, and fibromodulin in cartilage explants cultured under conditions in which aggrecan synthesis remains relatively constant.

Design: Articular cartilage was cultured in the presence or absence of 4 ng/ml TGF beta 1 for up to 16 days. Material extracted from cartilage was assayed for $^{35}SO_4$-large and small PGs and for total endogenous decorin, biglycan and fibromodulin.

Results: The synthesis of $^{35}SO_4$-small PGs increased during the 16 days in culture in response to TGF beta 1, but declined in control cultures. The difference in $^{35}SO_4$-decorin between TGF beta 1 and control samples reached nine-fold after 16 days, while the difference in total endogenous decorin was less than 1.5-fold. $^{35}SO_4$-decorin, which was present in TGF beta 1-treated cultures had an identical core protein, but a longer glycosaminoglycan chain than that of decorin in control cultures. No significant differences in endogenous biglycan were detected, but accumulation of fibromodulin in TGF beta 1 explants exceeded fibromodulin in controls, on average, by 3.8-fold. Fibromodulin was present in cartilage in both keratan sulfate- and non-sulfated oligosaccharide-substituted forms.

Conclusions: The accumulation of each of the three small PGs was affected to a different extent in response to TGF beta 1. Of the three, fibromodulin content was most rapidly augmented in response to TGF beta 1.
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Key words: Fibromodulin, Small proteoglycan, Osteoarthritis, Transforming growth factor beta.

Introduction

Decorin, biglycan, and fibromodulin are members of the small, leucine-rich proteoglycan (PGs) (SLRP) family of proteins and are located in articular cartilage as well as in other tissues[1,2]. Both decorin and fibromodulin have been localized by electron microscopy to collagen fibrils in carti-

lage. In *in vitro* studies, decorin and fibromodulin influence the rate and extent of collagen type I fibrillogenesis. A direct role in control of type I collagen fibrillogenesis in the tendon has been confirmed for fibromodulin by observation of fibromodulin-null mice[3] and for decorin, both by observations of decorin-deficient mice[4] and by disruption of decorin–fibronectin–collagen interactions *in vivo* using blocking peptides[5]. Thus, it is reasonable to think that the function of decorin and fibromodulin in cartilage is to regulate the diameter and organization of collagen fibrils, although this has not been directly confirmed.

Decorin, biglycan, and fibromodulin bind to active transforming growth factor (TGF) betas 1, 2 and 3 via their core proteins, with both high-affinity and low-affinity binding sites[6]. The chondroitin/dermatan sulfate chains of decorin and biglycan appear to somewhat hinder this binding, making TGF beta binding to fibromodulin more effective than binding to decorin or biglycan within the tissue. In the study by Hildebrand *et al.*[6], this binding was specific for TGF beta out of several other growth factors tested and suggests an important role for these small PGs in

regulating the activity of TGF beta on the synthesis of matrix components. Studies by Markmann et al.[7] suggest that decorin must be bound to matrix in order to sequester and inactivate TGF beta.

In turn, TGF beta contributes to the regulation of the synthesis of the small PGs. This has been studied in chondrocyte cell cultures. Several investigators have reported that TGF betas increase the synthesis of the small PGs in cultures of isolated chondrocytes. Decorin and biglycan have typically been the focus of these studies, but reports have been inconsistent as to whether one or the other, or both are elevated. Roughley et al.[8] reported a decrease in decorin mRNA in chondrocytes cultured with TGF beta in monolayers and Demoor-Fossard[9] reported that TGF beta inhibited decorin synthesis in chondrocyte monolayers, but not in chondrocytes in alginate beads. There is no information about the effect of TGF beta on fibromodulin in chondrocytes, with the exception of preliminary data showing two-fold increases in fibromodulin at the RNA and protein level in chondrocytes cultured in alginate beads[10].

Recent studies have focused on the SLRP in osteoarthritic cartilage. *In vivo*, Cs-Szabó et al.[11] have shown that mRNA for aggrecan, decorin, biglycan, and fibromodulin are increased in human knee osteoarthritis (OA), while Dourado et al.[12] reported similar increases for the canine anterior cruciate ligament transection model. Both laboratories reported greater changes in biglycan than in decorin (10:5.8 and 3.9:1.2, respectively). Fibromodulin mRNA was reported to increase by 11.5- and 2.4-fold, respectively. Cs-Szabó reported comparable increases at the protein level as well as for decorin, biglycan, and fibromodulin. Little et al.[13] reported increased decorin and biglycan synthesis in lateral tibial and femoral cartilage after a lateral meniscectomy in sheep. Most recently, Bock et al.[14] reported increased transcription and translation of both decorin and biglycan in late stage human OA, with highest levels in areas adjacent to the lesion. On the other hand, Poole et al.[15] reported no overall differences in biglycan and decorin contents in human knee OA, although loss from the superficial layers of osteoarthritic cartilage was greater.

A role for TGF betas and small PGs, possibly related, in OA progression is under consideration. TGF beta has been implicated in synovitis[16], in OA progression[17,18], and osteophyte formation. Furthermore, chondrocytes from OA cartilage may be more responsive to TGF beta than normal chondrocytes[19]. The accumulation of small PGs in human cartilage in late stage OA[11] would increase the potential to bind and sequester TGF beta and possibly mitigate effects on OA[6]. However, bound and sequestered TGF beta would fail to stimulate synthesis of the small PGs and other matrix components required to maintain a healthy cartilage. Thus, the consequences are really not known, and could be positive or negative outcomes for OA.

Studies that have been done to better define the relationship among TGF beta, chondrocyte synthesis of small PGs, and OA have not been altogether consistent, but at least some of the confusion must be attributed to the focus on chondrocytes in culture. Chondrocytes in monolayer rapidly dedifferentiate. Even chondrocytes in three-dimensional cultures fail to mimic the *in vivo* chondrocyte in all respects[20]. Explant cultures, with intact, native matrix, provide conditions closer to *in vivo*. Our laboratory has a well-characterized explant culture system in which aggrecan remains constant, but fibronectin, an early marker of degenerating cartilage, accumulates[21]. Preliminary data

suggested increased synthesis of total small PGs as well. Our goal was to determine the effect of TGF beta 1 individually on each of the small PGs, decorin, biglycan, and fibromodulin, in this system.

Materials and methods

MATERIALS

L-[^{35}S]methionine (1200 Ci/mmol) and Na$_2$^{35}SO$_4$ (25–40 Ci/mg) and L-[4,5-^{3}H] leucine were purchased from Amersham (Arlington Heights, IL). Peroxidase-linked goat anti-mouse IgG, and peroxidase-linked goat anti-rabbit IgG were purchased from Cappel Biomedicals (Westchester, PA). Hams F$_{12}$ and Gey's balanced salt solutions were from GIBCO (Grand Island, NY). ITSCR$^+$ serum-free supplement was from Collaborative Biomedical Products (Bedford, MA). Human recombinant TGF beta 1 was purchased from Life Technologies (Gibco, BRL) as powder lyophilized from 5 mM HCl containing 50 µg of human serum albumin per microgram of the cytokine and reconstituted with sterile H$_2$O. All storage vessels and pipette tips used to handle stock of TGF beta 1 were silanized.

Antibodies to canine biglycan and decorin[10] and cross-reactive purified polyclonal antibody raised against a C-terminal amino acid sequence of human fibromodulin[22] were prepared. Canine biglycan and decorin cDNAs were sequenced and specific peptide sequences were selected from the C-terminal of biglycan and decorin (Gene bank accession numbers: Canine biglycan, U83140; Canine decorin, U83141). Synthetic peptides were coupled with ovalbumin and injected to rabbits. Polyclonal anti-sera were purified on affinity columns on which the corresponding peptides were bound[10]. The anti-human fibromodulin antibody preparation has been described previously[22] and cross-reacted with canine fibromodulin. The anti-decorin monoclonal antibody, 6D6[23,24], used for immunoprecipitation, was the gift of Dr Paul Scott (Edmonton, Alberta, Canada). Nitrocellulose membranes were obtained from Osmonics (Westborough, MA). Hybond™–N$^+$ membranes were obtained from Amersham Pharmacia Biotech Limited (Little Chalfont, Buckinghamshire, England).

Methods

EXPLANT CULTURE

Source of explant cartilage

The sources of cartilage were Labrador Retrievers or Labrador Retriever–Greyhound crosses from a colony maintained at the James A. Baker Institute for Animal Health and bred for the study of genetic markers of canine hip dysplasia and its concomitant OA. Dogs ranged in age from 8 to 28 months.

Culture conditions

Cartilage was obtained at necropsy from Labrador retrievers from our colony of dogs with individuals at high and low risk for developing OA. Only macroscopically disease-free cartilage was removed aseptically from the hip, knee, or shoulder joints of these Labrador Retriever dogs and placed into Gey's balanced salt solution; however, some cartilage sampled may be predisposed for development of cartilage damage[25,26]. The cartilage was rinsed three times

and approximately 50 mg was placed into screw-capped vials or 24-well tissue culture dishes (Corning, NY) containing 2 ml of the appropriate culture medium. All explants were incubated in Hams F_{12} nutrient medium supplemented with ITSCR+ and with $CaCl_2$[21]. In some experiments, Na_2SO_4 was added to a concentration of 0.3 or 1.6 mM to be certain that available sulfate was not limiting for PG synthesis. Cartilage was cultured for 1 or 15 days with 4 ng/ml TGF beta 1, following 1–2 days of preincubation. Controls were cultured for the same length of time in the absence of TGF beta. The dosage chosen is physiologically relevant. TGF beta 1 levels in synovial fluids from OA patients varied widely in one study, reaching as high as 10 ng/ml and averaging to about 2.5 ng/ml[27]. Furthermore, 4 ng/ml of TGF beta was shown previously to stimulate aggrecan synthesis under the basal conditions[28], but not under the serum-free supplementation used in these experiments[21]. However, at this dosage, the synthesis of other proteins, especially fibronectin, is markedly affected[21]. In preliminary experiments, addition of vehicle alone had no effect on cartilage metabolism. Medium was changed every 3 or 4 days at which time fresh TGF beta was added. Radioactive precursors ($^{35}SO_4$ or ^{3}H-leucine plus ^{35}S-methionine) were added to the culture on the day before harvest. After an overnight incubation, explants were washed three times with Gey's balanced salt solution and frozen until further analyses.

EXTRACTION AND QUANTITATION OF TOTAL PGS

Cartilage was extracted with 4 M urea in 0.05 M phosphate buffer, pH 7.4, or with 4 M guanidinium chloride in 0.05 M acetate buffer, pH 5.8, in the presence of a cocktail of protease inhibitors (0.3 mM benzamidine, 20 mM ethylenediamine tetraacetate (EDTA), 10 mM *N*-ethylmaleimide, 0.4 mM phenylmethylsulfonylfluoride, final concentrations). To determine total PG synthesis, the cartilage residue after extraction was digested with 1 mg/ml papain in 0.05 M phosphate buffer containing 0.0326% *N*-acetylcysteine and 2 mM EDTA at 60°C for 4 h, and aliquots of digests and extracts were added to Ecoscint (National Diagnostics, Atlanta, GA) and quantitated in a Beckman LS6800 liquid scintillation counter. Total sulfated glycosaminoglycans (GAG) in the extracts and papain digests were determined by the dimethylmethylene blue (DMMB) assay of Farndale[29].

PURIFICATION, CHARACTERIZATION, AND QUANTITATION OF NEWLY SYNTHESIZED PGS

For characterization of $^{35}SO_4$-PG and ^{3}H/^{35}S-PG, urea and/or guanidinium chloride extracts were applied to a DEAE ion exchange column (DE52 cellulose) in 6 M urea, 0.05 M Tris–HCl, pH 7.5, 0.1 M NaCl. Columns were eluted with 0.2, 0.3, and 1.0 M NaCl in 6 M urea, 0.05 M Tris–HCl, pH 7.5, and the 0.3 and 1.0 M NaCl fractions were analyzed by gradient gel electrophoresis and detected by autoradiography or Western analysis.

Release of free GAG chains

To analyze free GAG chains, guanidinium chloride extracts were subject to gel filtration chromatography on Sepharose CL6B. Fractions corresponding to aggrecan and to the small PGs with GAG substitution were pooled separately, transferred to digest buffer and digested for 4 h at 60°C with 0.5 mg/ml papain. The samples were centrifuged and made 5% with potassium acetate in 95% ethanol. The $^{35}SO_4$-GAGs were precipitated over 2 days at 4°C and then washed with ethanol, dried, redissolved in distilled, deionized water, prepared for electrophoresis, and were detected by autoradiography.

Immunoprecipitation of decorin

The 0.3 M NaCl fractions from DEAE anion exchange columns were transferred to a dilution buffer (10 mM phosphate buffer, pH 7.2, containing 0.5% Na deoxycholate, 1% Triton X-100, 0.1% SDS, 100 mM NaCl, and 1 mM EDTA) and incubated overnight with monoclonal antibody 6D6 or with a control antibody at 4°C. The immune complex was precipitated with protein G agarose. Samples were then washed three times in 20 mM Tris buffer, pH 8.8, containing 0.5% Na deoxycholate, 0.5% NP 40, 50 mM NaCl, 2 mM PMSF, and 2 mM EDTA. Immunoprecipitated small PGs were resolved by SDS gradient gel electrophoresis[30] after heating at 95°C for 10 min in digestion buffer (50 mM Tris, pH 6.8, 2% SDS, 2 mM PMSF, and 2 mM EDTA), and were visualized by autoradiography.

PURIFICATION, CHARACTERIZATION, AND QUANTITATION OF ENDOGENOUS SMALL PGS

For purification, characterization, and quantitation of total decorin, biglycan, and fibromodulin, the guanidinium chloride extracts were directly analyzed by Western analysis before and after enzymatic digestion.

Enzymatic digestion

For the analysis of biglycan, decorin, and fibromodulin core proteins, samples of the extracts (20–40 µg dry weight) were dialyzed to 0.1 M Tris, 0.05 M Na-acetate, pH 7.3, and were digested with 20 mU proteinase-free chondroitinase ABC (Seikagaku) at 37°C overnight to remove chondroitin sulfate or dermatan sulfate side chains from the core proteins of decorin and biglycan. After adjusting the pH to 6.0, one-half of the samples were further digested with Keratanase II (Seikagaku) in the presence of proteinase inhibitors at 37°C overnight, to remove keratan sulfate (KS) side chains from the core protein of fibromodulin. One-half of this sample was further digested with *N*-glycanase [PNGase F; New England BioLabs, Inc. (Beverly, MA)] according to the manufacturer's instructions, to remove *N*-linked substitution from the core proteins of fibromodulin.

SODIUM DODECYL SULFATE–POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS–PAGE), AUTORADIOGRAPHY, AND WESTERN BLOTTING

Small PGs and their core proteins were separated in 12% uniform or 4–15% gradient slab gels under reducing conditions using the Tris/glycine buffer system described by Laemmli[31]. Gels were stained with Coomassie brilliant blue and/or toluidine blue, or were electrophoretically transferred onto nitrocellulose for free core proteins or onto Hybond–N+ nylon membranes for native PGs.

Autoradiography

Stained gels were dried and exposed to a phosphorimager screen. Radioactive PGs and core proteins were

Table I
Proteoglycans in cartilage explants cultured in the presence or absence of TGF beta 1

	Total $^{35}SO_4$-PG (cpm/mg wet weight)	$^{35}SO_4$-PG in urea (% of total)	Total PG (µg/mg wet)	PG in urea (% of total)
Control (16)	7139±3502	2.6±2.2	44.1±11.2	4.9±1.9
+TGF beta 1 (16)	7550±2644	8.1±2.3	37.3±8.4	6.4±2.5
	$P=0.542$	$P<0.001$	$P=0.056$	$P=0.012$

The data in this table compare total endogenous and newly synthesized PGs after 16 days in culture with endogenous and newly synthesized PGs that are easily extracted from articular cartilage with 4 M urea. Cartilage was cultured in the presence or absence of 4 ng/ml TGF beta 1. On day 15, cartilage was pulsed for 1 day with $Na_2{}^{35}SO_4$. At harvest, cartilage was extracted with urea and the residue digested with papain. An aliquot of each urea extract and papain digest was counted in a liquid scintillation ($^{35}SO_4$-PG) counter or assayed for GAG content with the DMMB assay (PG). The data represent four different experiments (four different donor animals), with four replicates from each experiment, such that the number in parenthesis indicates the total number of replicates for each group. The data were analyzed by two-way ANOVA, general linear model, with respect to dog and treatment.

quantitated using MACBAS software (Fuji Medical Systems USA, Stamford, CT) after scanning in the Fujix B_{10}-Imaging Analyzer.

Western blot hybridization

Membranes containing transferred proteins were blocked with 5% milk in Tris buffered saline containing Tween 20, and were probed with antibodies to decorin, biglycan, or fibromodulin followed by peroxidase-labeled goat anti-rabbit IgG and visualized by enhanced chemiluminescence assay (ECL) followed by quantitation of the signal on ECL films by a BioRad image analyzer.

STATISTICAL METHODS

Results were analyzed by ANOVA, general linear model, using MINITAB software (Kent, Boston, MA). Significance was set at $P<0.05$.

Results

PROTEOGLYCAN SYNTHESIS IN CARTILAGE EXPLANTS

When cartilage explants were cultured in the presence of TGF beta 1 and supplemented with insulin and calcium, total $^{35}SO_4$-PG was unchanged or only modestly increased, but $^{35}SO_4$-PG in a 4 M urea extract was increased several folds with respect to control cultures (Table I). Analysis of urea extracts by gel filtration chromatography, DEAE chromatography, and gradient gel electrophoresis showed that $^{35}SO_4$-small PG are predominant in urea extracts, although additional material, presumably aggrecan, is present at the top of the gel (data not shown). Further analysis was performed on both urea and guanidinium chloride extracts of cartilage explants in an attempt to characterize the effect of TGF beta1 on synthesis and accumulation of the small PGs, decorin, biglycan, and fibromodulin.

CHARACTERIZATION AND QUANTITATION OF NEWLY SYNTHESIZED SMALL PGS BY AUTORADIOGRAPHIC ANALYSIS

After 2 days in culture, control cartilage synthesized $^{35}SO_4$-small PGs or $^3H/^{35}S$-small PGs, which co-migrated with endogenous decorin and biglycan identified by Western analyses (data not shown). Decorin was the predominant small $^{35}SO_4$-PG and eluted from DEAE anion exchange columns with 0.3 M NaCl (Fig. 1, lanes 4 and 6). Explant culture was continued for an additional 14 days in

Small proteoglycans in control and TGF beta1 treated cartilage explants separated on gradient SDS-PAGE before and after chondroitinase ABC digestion
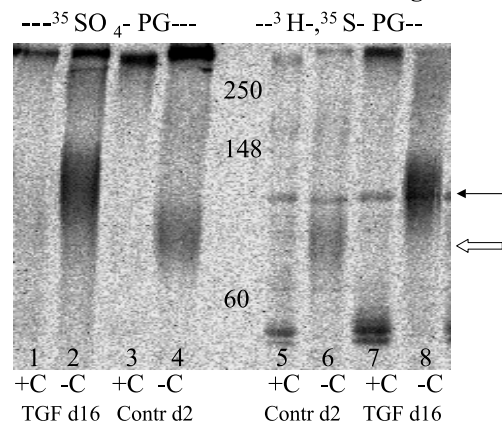

Fig. 1. Autoradiograph of small PGs in control and TGF beta 1-treated cartilage explants. This figure contrasts newly synthesized small PGs in control cultures after 2 days with small PGs in TGF beta 1-treated cultures after 16 days. Small PGs were purified by DEAE anion exchange chromatography and analyzed by SDS/PAGE and autoradiography. From left to right, lanes 1–4 show anion exchange fractions of proteins labeled with $^{35}SO_4$-GAG and lanes 5–8 show anion exchange fractions of proteins labeled with a combination of ^3H-leucine and ^{35}S-methionine. The numbers between lanes 1–4 and 5–8 indicate the positions of molecular weight markers. Fractions in lanes 1, 3, 5, and 7 were digested with chondroitinase ABC prior to electrophoresis (+C). Fractions in lanes 2, 4, 6, and 8 were intact (–C). Lanes 1, 2, 7, and 8 are 1.0 M NaCl DEAE fractions from 16-day TGF beta 1-treated cultures (TGF d16). Lanes 3, 4, 5, and 6 are 0.3 M NaCl DEAE fractions from 2-day control cultures (Contr d2). Increased synthesis of small PG in response to TGF beta 1 is apparent (compare lane 2 with lane 4, and lane 8 with lane 6). The data also show that both small PGs in day 2 control cultures, and the highest molecular weight small PGs in day 16 TGF beta-treated cultures were completely digested by chondroitinase ABC (see lanes 1 and 3, and lanes 5 and 7), leaving core proteins of identical molecular weight (compare lane 5 with lane 7). The open arrow points to intact decorin. The closed arrow points to intact, high molecular weight decorin synthesized in the presence of TGF beta 1.

the presence or absence of TGF beta 1. By day 16, synthesis of $^{35}SO_4$-small PGs was increased in TGF beta-treated samples. A three-fold increase in total small PG synthesis relative to day 2 controls (4471/1566, Table II), and as much as a nine-fold increase relative to day 16

Table II

$^{35}SO_4$-decorin in articular cartilage explants cultured in the presence or absence of TGF beta 1

	Decorin (cpm/mg wet)	HMW Decorin[*] (cpm/mg wet)
Control		
Day 2	1566	—
Day 16	370	124
+TGF beta 1		
Day 2	1120	754
Day 16	729	4042

This table presents an analysis of newly synthesized decorin in control and TGF beta 1-treated cultures on day 2 and on day 16, both with respect to quantitative differences and with respect to molecular weight. Cartilage was pulsed overnight on day 1 and on day 15. Urea extracts were purified by DEAE anion exchange chromatography, eluted with 0.3 and 1.0 M NaCl, and further separated by 5–15% SDS gradient gel electrophoresis. Dried gels were quantitated using a phosphorimager and MACBAS software yielding relative amounts of newly synthesized decorin. Differences between control and TGF beta 1-treated cultures were minimal at day 2, although some HMW decorin is already apparent in cultures treated with TGF beta 1. By day 16, decorin synthesis declined in controls, although some HMW decorin was detected. In contrast, synthesis of HMW decorin dramatically increased in TGF beta 1-treated cultures.

[*]HMW decorin has a mobility on SDS gels that is less than that of decorin from controls.

controls (4471/494, Table II), were noted. Furthermore, much of the small PG synthesized after 16 days of culture in the presence of TGF beta 1 had a higher molecular weight (HMW) than that of decorin found in the controls (Fig. 1, lanes 2 and 8; Table II). It was extracted from cartilage by either urea or guanidinium chloride, and was eluted from DEAE anion exchange columns in both the 0.3 and 1.0 M NaCl fractions. Although $^{35}SO_4$-small PG synthesis decreased in the controls, a small shift in molecular weight was also evident (Table II). The GAG chains of the HMW small PG synthesized in the presence of TGF beta 1 were sensitive to chondroitinase ABC digestion (Fig. 1, lanes 1 and 7), and the core proteins were identical in electrophoretic mobility to those obtained from the small PGs synthesized in the controls (Fig. 1, lanes 5 and 7).

The data are consistent with identification of the HMW $^{35}SO_4$-PG as decorin with a longer GAG chain. The newly synthesized, HMW small PG could be immunoprecipitated by an anti-decorin antibody (not shown). Electrophoretic analysis of the free GAG chains after papain digestion indicated that GAG chains on the small PGs from TGF beta-treated samples ranged from 25 to 50 kDa and, on average, were 1.5 times the size of the GAG chains from the small PGs synthesized in day 2 controls and twice the size of the GAG chains from all aggrecans (not shown). Furthermore, when synthesis of core proteins were followed with radioactive amino acid precursors, an increase in total small PG synthesis was confirmed. The increase in $^{35}SO_4$-GAG in response to TGF beta exceeded the increase in $^3H/^{35}S$-core protein by 1.4-fold, entirely consistent with an increase in GAG chain length in addition to a total increase in synthesis (not shown).

$^{35}SO_4$-biglycan, which eluted from DEAE columns with both 0.3 and 1 M NaCl was present at lower levels than decorin, and was not always clearly separated from the top of the gel. It was not further characterized. Fibromodulin substituted with KS has a mobility similar to decorin. Little

or no ^{35}SO-PG or $^3H/^{35}S$-PG remained after digestion with chondroitinase ABC (Fig. 1), suggesting that newly synthesized fibromodulin was below detectable levels in these experiments. However, N-linked oligosaccharide-substituted forms of fibromodulin lacking sulfate would elute from DEAE columns at lower salt concentrations, less than 0.3 M. Although small amounts of $^3H/^{35}S$-proteins consistent with N-linked oligosaccharide-substituted fibromodulin were found in the 0.2 M NaCl fraction, they were not positively identified.

CHARACTERIZATION AND QUANTITATION OF TOTAL ENDOGENOUS SMALL PGS BY WESTERN ANALYSIS AND CHEMILUMINESCENCE

Characterization by size and extent of glycosylation

The total decorin present after 16 days in control and TGF beta 1-treated cultures were very similar in mobility when identified by an anti-decorin antibody in Western analysis (Fig. 2, panel 1), although small amounts of a HMW decorin were detected by Western analysis in TGF beta 1-treated cultures in some experiments (Fig. 2, panel 1, lanes 3 and 4, arrowheads). This decorin approached the size of the newly synthesized decorin revealed by isotope labeling. After digestion with chondroitinase ABC, the sizes of the core proteins from control and TGF beta-treated cultures were identical (Fig. 2, panel 2), consistent with data shown for newly synthesized decorin in Fig. 1. Most decorin was found with the GAG attached, although a small amount of decorin-free core protein was detected. In contrast, both native biglycan, with GAG attached as well as free-biglycan core protein, were detected in explanted cartilage (data not shown).

Native fibromodulins consisted of a diffuse population of molecules ranging from approximately 60 to 100 kDa as is the characteristic of KS substitution (Fig. 3, panel 1), and also of a more discrete population near 66 kDa, which can be seen more clearly when the native molecule is blotted to nitrocellulose (Fig. 3, panel 2). When sulfated GAGs (KS) were removed by enzymatic digestion from the core protein, a more compact band was detected at 66 kD indicating that the original sample did contain a subpopulation of the core protein which was substituted with KS (Fig. 3, panel 3). However, further removal of N-linked substituents from the core protein with N-glycanase revealed that the majority of the core protein was substituted with N-linked oligosaccharide. After this step, fibromodulin was detected as a major band at 46 kD and a minor band at 40 kD (Fig. 3, panel 4). These species were not detected in the native extract; thus, fibromodulin is present in substituted KS-, and N-linked oligosaccharide-substituted forms, but not as a free-core protein. The total amounts of fibromodulin changed as a result of TGF beta 1 treatment, but the substitution of fibromodulin was similar in both control and TGF beta 1-treated cultures.

Quantitation of total endogenous small PGs

For quantitation of total accumulated endogenous small PGs (native as well as free-core protein), GAG chains were removed from decorin and biglycan with chondroitinase ABC and from fibromodulin with keratanase II, and the resulting core proteins were blotted to nitrocellulose followed by Western analysis [Figs. 2 (panel 2) and 3 (panel 3)], with quantitation using ECL (Figs. 4 and 5). No significant changes in decorin and biglycan contents from day 2 to day 16 were detected in control cultures (Fig. 4). No

Western analysis of decorin

Hybond N+; w/o Digestion
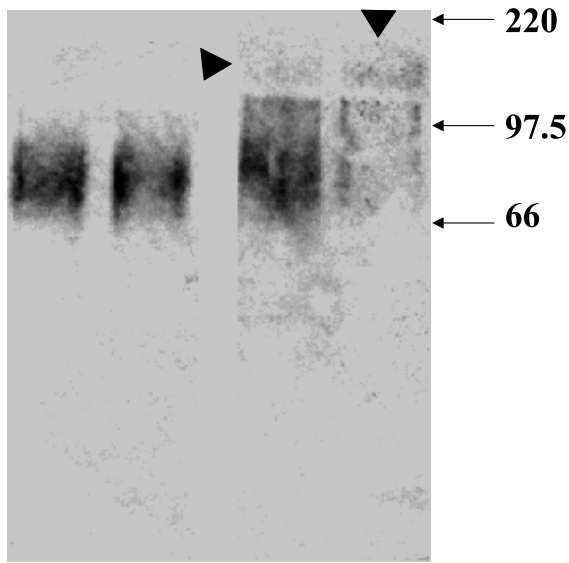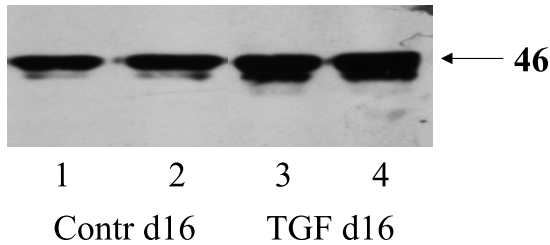

Nitrocellulose; Chondroitinase ABC

Fig. 2. Western analysis of decorin in control and TGF beta 1-treated cartilage. Decorin present in cartilage extract was analyzed by SDS/PAGE and immunoblotting as the native molecule (panel 1) and after removal of GAG chains by chondroitinase ABC digestion (panel 2). Lanes 1 and 2 (duplicates) analyze 16-day control cultures. Lanes 3 and 4 (duplicates) analyze samples from TGF beta 1-treated samples after 16 days in culture. Native samples were blotted to a positively charged membrane (Hybond–N+, panel 1). Core proteins, resulting from digestion with chondroitinase ABC, were blotted to nitrocellulose (panel 2). The blots were probed with a rabbit polyclonal antibody raised against decorin C-terminal peptide sequences. The arrowheads in panel 1 point to a modest accumulation of HMW decorin in TGF beta 1-treated culture.
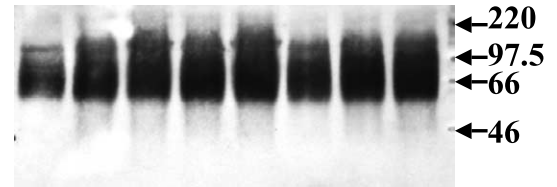
Western analyses of fibromodulin

Hybond N+; w/o Digestion

Nitrocellulose; w/o Digestion

Nitrocellulose; Keratanase II

Nitrocellulose; Keratanase II + N-glycanase

Fig. 3. Western analysis of fibromodulin in control and TGF beta 1-treated cartilage. Fibromodulin present in cartilage extracts was analyzed with a series of enzymatic digestions as described in section Methods, in order to evaluate the extent of substitution with KS and non-sulfated oligosaccharides. Analysis was done by SDS/PAGE and immunoblotting. Lanes 1, 2 (duplicates) and 5, 6 (duplicates) are controls. Lanes 3, 4 (duplicates) and 7, 8 (duplicates) are TGF beta-treated. Lanes 1–4 analyze 2-day cultures. Lanes 5–8 analyze 16-day cultures. Note: the band that appears in all blots above 100 kD is non-specific. Native PGs were blotted to positively charged nylon (panel 1) or nitrocellulose (panel 2). Nitrocellulose was also used for blotting following digestion of native PGs with chondroitinase ABC and keratanase II (panel 3) plus *N*-glycanase (panel 4). KS-substituted GAGs (wide bands) can be seen in panel 1, and more faintly in panel 2. Fibromodulin, after removal of KS, has a molecular weight of 66 kDa (panel 3), indicating substitution with *N*-linked constituents, which can be removed by *N*-glycanase, leaving the core proteins (panel 4). Fibromodulin is present in partially KS-, partially non-sulfated oligosaccharide-substituted forms, but not as a free-core protein.

significant changes were detected for fibromodulin either, but sample number (*n*=2) was limited. A small, but significant increase in decorin was detectable after 16 days of culture in the presence of TGF beta 1 (Fig. 5). No significant change in biglycan was detected in response to TGF beta 1 (Fig. 5). Increases in fibromodulin in response to TGF beta 1 were the most extensive averaging 3.8-fold (Fig. 5).
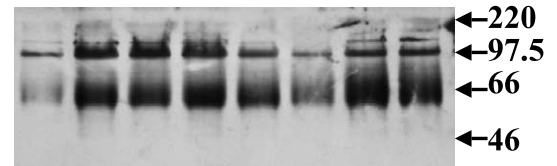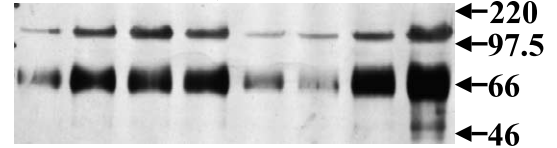
Discussion

Our goal was to study the effects of TGF beta 1 on the synthesis of three small PGs, decorin, biglycan, and fibromodulin by chondrocytes. To accomplish this, we chose the conditions as close as possible to *in vivo* conditions by using cartilage explants with an intact matrix, rather than isolated chondrocytes in culture. We focused on small PGs, specifically, by choosing culture conditions in which aggrecan synthesis was not, or was only minimally, affected[21]. Under these culture conditions, TGF beta 1 induces a marked increase in fibronectin synthesis, which is one characteristic of OA cartilage[21]. Thus, in these experiments, we looked at the effect of TGF beta 1 on small PGs
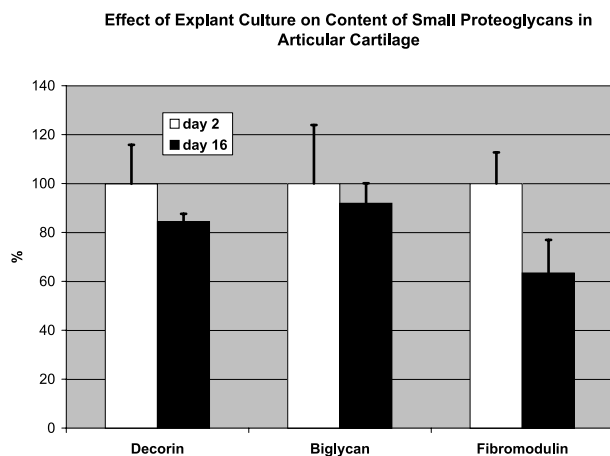
Fig. 4. Effect of explant culture on content of small PGs in articular cartilage. The figure represents a quantitative comparison of each of the small PGs in control cultures between 2 days (white bars) and 16 days (black bars) of incubation. At days 2 and 16, cartilage was collected and extracted with guanidinium chloride. Extracts were digested with chondroitinase ABC, or chondroitinase ABC followed by keratanase II, prior to electrophoresis on 12% SDS gels, and blotting to nitrocellulose membranes. The blots were probed with rabbit polyclonal antibodies and immunoreactive material was detected with ECL. Results of quantitation of the signal on the film are presented graphically. The average of all replicates for day 2 control cultures was set to 100. The data are presented as mean±standard deviation. ($n=4$ for decorin and biglycan; $n=2$ for fibromodulin). No significant change with time was noted.
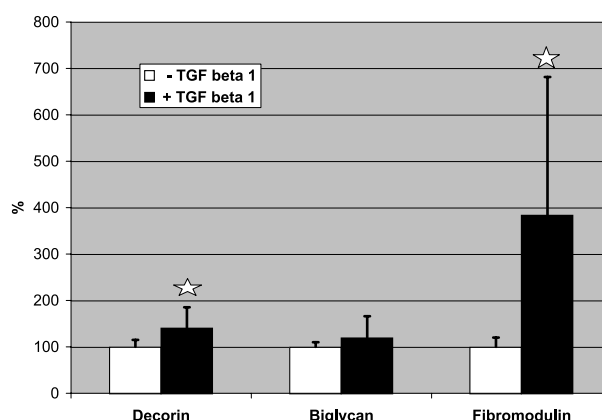

Fig. 5. Accumulation of decorin, biglycan, and fibromodulin in articular cartilage in response to TGF beta 1. The figure represents a quantitative comparison of each of the small PGs after 16 days in culture in the absence (white bars) and in the presence (black bars) of TGF beta 1. Core proteins were analyzed by Western blotting as described inFig. 4. Results of quantitation are presented graphically for day 16 samples. Data were obtained from two different experiments for decorin and biglycan and from three experiments for fibromodulin. The average of all replicates for day 16 control cultures was set equal to 100 in each experiment. The data are presented as mean±standard deviation ($n=7$ for decorin and biglycan; $n=8$ and 7, respectively, for fibromodulin). *indicates differences that were significant at $P<0.05$.

under conditions which support some of the characteristics of the early OA phenotype. The entire cartilage used in this study was disease-free, but since donor animals came from a colony in which dogs of similar age and breed do develop osteoarthritic lesions, the cartilage may have been predisposed to develop OA. The source of this cartilage may facilitate comparison of these results with OA cartilage in the future. For the first time, we report a 3.8-fold increase in fibromodulin in canine articular cartilage in response to TGF beta 1, and we show that fibromodulin in canine articular cartilage is partially substituted with KS.

The major newly synthesized small PG in cultures treated with TGF beta 1 had a greater molecular weight than endogenous decorin. This at first led to the hypothesis that cartilage explants synthesized a novel CS/DS small PG in response to TGF beta[32]. Subsequently, we identified the HMW small PG as decorin. First, the HMW small PG could be immunoprecipitated with an anti-decorin antibody, which is specific for decorin with no cross-reactivity with biglycan or fibromodulin in immunoblots[24]. Second, the HMW small PG has GAG chains 1.5-fold larger than those found in endogenous decorin, sufficient to account for the increase in molecular weight. To explain the HMW small PG, we could have postulated the presence of biglycan with a single GAG chain. However, although biglycan with a single GAG chain has a size similar to HMW decorin, we observed no evidence for co-precipitation of any biglycan with decorin in our experiments. Furthermore, it is not necessary to postulate biglycan with a single GAG chain to account for our results, which can also be explained by the longer GAG chains on decorin. The effect of TGF beta on GAG chain length has been reported previously; e.g., Chan and Anastassiades[33] reported an increase in length of chondroitin sulfate chains synthesized in the presence of

TGF beta in high density bovine articular chondrocyte cultures.

In our experiments, the majority of newly synthesized small PGs in both control and TGF beta 1-treated explants was decorin. Some biglycan was detected. Newly synthesized, KS-substituted fibromodulin was below detectable levels. This is consistent with the findings of Roughley[34] that decorin synthesis greatly exceeds biglycan synthesis in adult humans and with the ratio of mRNAs reported by Cs-Szabó et al.[11] for adult human cartilage (decorin: biglycan:fibromodulin=158:8:1). It is also consistent with other experiments in our laboratory in which we have isolated pure decorin from large quantities of articular cartilage. Our estimated yield of 700 µg/g wet weight of tissue agrees well with reports of Rosenberg et al.[35]. Preliminary estimates put biglycan levels at 25–40% and fibromodulin levels at 7–15% of decorin.

The three- to four-fold increase in $^{35}SO_4$-decorin including 1.5-fold increase in chain length in response to TGF beta 1 from day 2 to day 16, with shift towards HMW decorin, had little or no impact on total endogenous decorin over this same time-period. Likewise, in control cultures, synthesis of decorin declined from day 2 to day 16, but no net decrease in total decorin content was observed by day 16 (Fig. 4). Thus, we must consider whether or not newly synthesized decorin in culture and endogenous decorin represent the same metabolic pool, and/or whether the amount of decorin synthesized and lost during 2 weeks in culture represents only a small proportion of endogenous decorin. A half-life of 18 days has been reported for decorin in bovine collateral ligament[36], but additional work is needed to establish this parameter for articular cartilage.

Endogenous fibromodulin content was more responsive to culture with TGF beta than decorin and biglycan

contents. In one experiment, a difference of as much as seven-fold was noted. The greater net changes to fibromodulin mean that TGF beta can affect the relative composition of the small PGs with respect to each other. If fibromodulin has a role in fibrillogenesis of collagen type II, as has been demonstrated in transgenic mice for collagen type I fibrillogenesis[3], changes in the stoichiometry of fibromodulin with respect to the other small PGs is likely to have significant consequences.

KS chains are present on fibromodulin from bovine cartilage obtained from cows from 3 months to 8 years of age[37]. Fibromodulin in the human fetus and neonate is a diffuse population of molecules between 70 and 110 kDa, consistent with KS substitution. In the human adult, the electrophoretic pattern becomes more discrete at 67 kDa. This corresponds to fibromodulin devoid of KS, and in some instances, mature adult forms exist with neither KS nor the non-sulfated polylactosamine[22,34]. Cartilage in this study came from the dogs that could be characterized as adolescent or young adult. Native fibromodulin with KS substitution as well as fibromodulin lacking KS were present in this cartilage. This is consistent with the reported composition of human cartilage of comparable age[38,39]; however, a function for these endogenous free-core proteins in cartilage has not been described.

Morales *et al.*[40] reported that, *in vivo*, articular cartilage contains a pool of TGF beta in excess of the amount of exogenous, active TGF beta that must be added to explants to affect the PG synthesis. However, most of this pool is sequestered and not readily accessible to the chondrocyte. The release and activation of this reserve after matrix degradation would allow for a role for TGF beta in the progression of OA and could account for osteophyte formation[17,19]. Does this postulated release and activation of TGF beta actually occur in OA cartilage *in vivo*? If so, over the extended time it may take for TGF beta 1-induced changes in the cartilage matrix to accumulate, we would expect to see elevated fibromodulin levels and a HMW decorin in OA cartilage *in vivo*. In fact, increased fibromodulin is observed in canine spontaneous and ACL transection models, as well as in human cartilage[41]. One specimen consisting of visually intact knee cartilage taken from a canine knee joint that had extensive lesions did contain a HMW decorin. However, two mild OA lesions showed no evidence of a change in the size of decorin small PG. This question will require further study.

An association of TGF beta with the progression of OA is not intuitive. One expects an anabolic factor, such as TGF beta to contribute to matrix repair. TGF beta should antagonize catabolic agents and restore aggrecan synthesis. However, an increase in the synthesis of matrix components may not be beneficial if the appropriate balance cannot be maintained. Changes in the ratio of the small PGs in cartilage, with respect to each other and to aggrecan, could have a profound influence on the pathogenesis of OA. For example, changes in the cartilage composition of decorin and/or fibromodulin, which affect fibril diameter could account for an early observation by Lust *et al.*[42], who reported that there is an absence of large diameter collagen fibrils in OA lesions in cartilage from dogs with canine hip dysplasia. Only medium and small diameter fibrils remain. Furthermore, fibronectin, including the cartilage-specific $(V+C)^-$ fibronectin isoform, is also elevated in OA cartilage[43,44]. This isoform was demonstrated to bind more decorin and biglycan than equivalent amounts of other

isoforms[45]. Thus, it is possible that fibronectin–small PG complexes exist in OA cartilage to a degree in which they do not occur in disease-free cartilage, and this could influence matrix organization. Fibronectin–decorin interactions influence fibrillogenesis of type I collagen[5] and might have a similar role with respect to type II collagen. It is also interesting to note that sites capable of binding alpha 4 beta 1 integrins and small PGs have been found in close proximity on fibronectin, suggesting functional cooperativity[46]. Chondrocytes from OA cartilage acquire expression of the alpha 4 beta 1 integrin not normally present[47]. These cell and matrix changes are likely to be reflected in changes in gene expression in OA cartilage, but this remains speculation at this point.

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