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Mechanical Loading and TGF-β Regulate Proteoglycan Synthesis in Tendon

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Fibrocartilage is found in tendon at sites where the tissue is subjected to transverse compressive loading *in vivo*. A significant characteristic of the tissue transition from tendon to fibrocartilage in bovine deep flexor tendon is increased gene expression, synthesis, and accumulation of both a large proteoglycan, aggrecan, and a small proteoglycan, biglycan. In order to investigate the cellular events involved in this response, segments of fetal bovine deep flexor tendon were subjected *in vitro* to cyclic compressive load for 72 h. Following loading, the level of aggrecan mRNA in cells from loaded tissue was increased 200–450% compared to matched nonloaded tissue segments, as determined by slot-blot analysis. The level of biglycan mRNA increased 100%, and the level of versican mRNA increased 130% in the loaded tissue. The level of decorin mRNA remained virtually unchanged, while expression of α1(I) collagen increased only 40%. When tissue segments were cultured in the presence of transforming growth factor (TGF)-β1 (1 ng/ml), the synthesis and expression of mRNA for both aggrecan and biglycan increased, whereas decorin expression was not affected. Similarity in both the direction and the pattern of the cellular response to mechanical load and TGF-β suggested a causal relationship. Both loading of tendon segments and TGF-β treatment increased expression of mRNA for TGF-β by ~40% compared to control tissue. In addition, the amount of newly synthesized TGF-β immunoprecipitated from extracts of loaded tissue was several-fold greater than that from nonloaded tissue. The experiments of this study support a hypothesis suggesting that one aspect of the response of cells in fetal tendon to compressive load is increased TGF-β synthesis which, in turn, stimulates synthesis of extracellular matrix proteoglycans and leads toward fibrocartilage formation.

Fibrocartilage develops in bovine deep flexor tendon at the point where the tendon wraps under sesamoid bones in the metatarsophalangeal joint of the foot (1). Throughout the purely tensional regions of this tendon the extracellular material consists of linear bundles of collagen and contains very little proteoglycan, most of which is decorin (2). The fibrocartilaginous region, in contrast, is composed of collagen fibers with a more random, or network, arrangement and contains a significant amount of interfibrillar matrix that stains intensely with Alcian blue, reflecting the presence of high levels of proteoglycan (3, 4). Compared to cells in the tensional region of tendon, the pattern of mRNA expressed by cells in the fibrocartilaginous region of adult tendon shows elevated levels of mRNA for aggrecan (the large proteoglycan expressed in cartilage), biglycan, and type II collagen (5). In fact, the pattern of mRNA expression in cells from the fibrocartilaginous region of adult tendon is nearly identical to the pattern expressed by articular chondrocytes. In contrast, cells in near-term fetal tendon expressed low levels of aggrecan mRNA and high levels of mRNA for type I collagen and decorin. In the compressed region of tendon, therefore, there is a change in extracellular matrix gene expression from a fibroblast-like pattern to a chondrocyte-like pattern, and this occurs after birth of the animal. This developmental change includes increased expression of the genes for both aggrecan and biglycan as well as a switch from type I to type II collagen gene expression.

Changes in proteoglycan accumulation in tendon have been previously shown by both *in vivo* and *in vitro* experiments to be correlated with the type of mechanical loading to which the tendon is subjected (6–8). For example, the glycosaminoglycan level was highest at

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the point where rabbit flexor digitorum profundus tendon wraps under the calcaneus. However, when normal compressive loading was relieved by surgical translocation of the tendon, the level of glycosaminoglycan in the unloaded tissue diminished rapidly (6). Replacement of the tendon to its original location, which reestablished transverse compressive loading of the tendon, resulted in a slow increase in its glycosaminoglycan content (6). Application of cyclic, uniaxial compressive loading to cultured explants of adult tendon fibrocartilage maintained the synthesis of large proteoglycans whereas synthesis of large proteoglycans was greatly diminished in matched explants cultured without loading (8). In addition, fetal tendon explants in culture responded to a 72-h cyclic compressive loading regimen with increased synthesis and mRNA levels for both aggrecan and biglycan (9). However, synthesis of decorin, the most abundant proteoglycan made by fetal tendon, was not regulated by mechanical loading. These experiments suggest that cells in tendon respond to cyclic compressive load with increased synthesis of aggrecan and biglycan, thus creating an extracellular matrix with a composition more like that found in adult tendon fibrocartilage.

The cellular mechanisms involved in regulation of proteoglycan synthesis by mechanical loading remain largely unknown. Coordinated changes in synthesis and degradation of extracellular matrix molecules, changes that could ultimately affect the composition of a tissue, are stimulated by cytokines (10). One cytokine, transforming growth factor-β (TGF-β),[3] has been specifically implicated as an important factor regulating cartilage-specific gene expression during development (11–14). A few studies have correlated the *in vivo* response of tissue to chemical insult with increased TGF-β expression and increased synthesis of extracellular matrix (15, 16). Increased release of TGF-β activity was induced in cultured cells by both intermittent hydrostatic pressure (17) and shear-stress loading (18). The current study explores the possibility that the coordinated change in proteoglycan synthesis induced in tendon by mechanical load involves regulation by the cytokine TGF-β.

MATERIALS AND METHODS

Tissue preparation. Fresh deep flexor tendons from the hind legs of near-term (7–9 months) bovine fetuses were obtained from a local slaughterhouse. Paired segments 8 mm long and approximately 4.5 mm thick × 10 mm wide were prepared from the region of tendon that will become fibrocartilaginous in the adult animal, as described previously (9). The wet weight of one segment was ~200 mg; dry weight was ~60 mg. The anterior–posterior thickness of each segment was measured and the tissue placed into culture in McCoy's 5A medium supplemented with 10% newborn calf serum (NCS, Gibco, Gaithersburg, MD), 5 U/ml penicillin/streptomycin, and 0.2 g/liter L-glutamine in a 5% CO_2 atmosphere. Following a 3-day culture period, paired tendon segments were either subjected to mechanical loading or treated with TGF-β1. Unless otherwise mentioned, all reagents used in this study were purchased from Sigma Chem. Co. (St. Louis, MO).

TGF-β1 treatment. Segments of fetal tendon were cultured for 3 days in medium containing 10% serum and then transferred to medium containing 10%, 1%, or no serum, with or without added TGF-β_1 (1 ng/ml, from porcine platelets, R&D Systems, Inc. Minneapolis, MN) and cultured for an additional 72 h. During the final 12 h of the culture period, $Na_2[^{35}S]O_4$ (50 μCi/ml, Dupont NEN, Boston, MA) was added. The radiolabeled proteoglycans were extracted and quantitated after separation by 5.5–20% gradient SDS/polyacrylamide gel electrophoresis, as previously described (9).

Mechanical loading. Six paired tendon segments were transferred to a loading device, as described by Evanko and Vogel (9). An impulse load was applied at a frequency of 1 cycle every 6 s (0.17 Hz) with a load duration of 0.2 s. One segment of each matched pair was cyclically loaded to 30% strain, based on the original thickness of the fresh tendon, for 24 or 72 h. The direction of applied load was perpendicular to the long axis of the tendon and approximated the pattern of loading of this tissue *in vivo*. The other segment of each pair was cultured in the same chamber without loading. Medium was changed every 24 h.

RNA purification, Northern blot, and slot-blot analysis. Following either mechanical loading or TGF-β1 treatment the tendon segments were minced with sterile razor blades and placed into Puck's saline. The minced tissue pieces of replicate treatments were combined and digested at 37°C in 0.75% Pronase (Calbiochem, San Diego, CA) in 40 ml of McCoy's 5A medium for 1 h. Following this, the tissue was digested in 0.15% collagenase (Worthington Biochemical Co., Freehold, NJ) in 40 ml of McCoy's 5A medium for 6 to 8 h, until the extracellular matrix was completely solubilized. The yield of cells was ~50 × 10^6 cells/g wet wt of tissue. There was no difference in the cell yield from control compared to loaded tissue. Total RNA was prepared from isolated cells using the acid guanidinium thiocyanate phenol–chloroform method of Chomczynski and Sacchi (19). Yield of total RNA was 1.1–1.4 μg RNA/10^6 cells or ~60 μg RNA/g wet wt of tissue. Cells and RNA were isolated from control and treated tissue at the same time, to diminish concern about confounding changes in mRNA level during the collagenase digestion period. It appears that mRNA levels in connective tissue cells can be quite stable. In one example, the total RNA yield from rabbit ligament and tendon, as well as levels of mRNA expression for several matrix molecules and housekeeping genes, was the same when RNA was isolated immediately after sacrifice as when it was isolated after the tissue had been stored for 96 h at 4°C (20).

Eight micrograms of each RNA preparation was used for Northern blot analysis of collagen and proteoglycan mRNA, while 16 μg was used to assess TGF-β mRNA expression. The RNA was denatured for 15 min at 60°C, electrophoresed through a 1% agarose/formaldehyde denaturing gel, and then capillary blotted onto nitrocellulose. For slot blot analysis, dilutions of total RNA (8 μg, 4 μg, 2 μg) were applied to nitrocellulose. All hybridizations were carried out for 18 h at 42°C in a solution containing 50% formamide (Bethesda Research Labs, Bethesda, MD), 5× saline–sodium citrate (SSC), 10% dextran sulfate, 100 μg/ml yeast RNA, 25 mM sodium phosphate, 0.02% Ficol 400 (Pharmacia, Piscataway, NJ), 0.02% bovine serum albumin, and 100 μg/ml salmon sperm DNA. Following hybridization the blots were washed two times at 54°C in 1× SSC/0.1% sodium dodecyl sulfate (SDS) for 30 min and two times at 54°C in 0.1× SSC/0.1%

[3] Abbreviations used: TGF-β, transforming growth factor-β; SSC, saline–sodium citrate; SDS, sodium dodecyl sulfate; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PMSF, phenylmethylsulfonyl fluoride; IP, immunoprecipitation; NCS, newborn calf serum; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay.

SDS for 30 min. Blots hybridized with the type I collagen probe were washed in a similar manner, but at 65°C. Hybridization signals were detected by autoradiography or utilizing the Molecular Dynamics phosphoimaging system (Molecular Dynamics, Sunnyvale, CA). Signal quantitation was performed by digitizing each of the autoradiographs with the aid of the Image-1 computer software package (Universal Imaging Co., Westchester, PA). The ImageQuant software package (Molecular Dynamics, Sunnyvale, CA) was used when signals were detected with the phosphoimaging system. Signal intensity for each of the mRNA transcripts was normalized to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) expression. The following cDNA clones were used: pHCAL1U (21), for α1(I) collagen; LA-5 (22), for aggrecan; P16 (23), for biglycan; PG20 (24), for decorin; a cDNA clone including nucleotides 71 to 1053 for glyceraldehyde-3-phosphate dehydrogenase (Clontech Inc., Palo Alto, CA); and a probe for human TGF-β1 (R&D Systems, Minneapolis, MN). Two mouse cDNA clones for detection of TGF-β2 and β3 mRNAs were provided by Dr. D. Danielpour (Bethesda, MD). Isolated DNA inserts (20 ng) were radiolabeled by random priming. Whole plasmid DNA (P16 cDNA clone, 0.2 μg) was radiolabeled by nick translation. Both reactions were carried out in the presence of [^{32}P]dCTP utilizing kits purchased from Pharmacia (Piscataway, NJ).

Radiolabeling and extraction of proteoglycans and TGF-β. Following mechanical loading, tissue was immediately transferred to individual wells of a 24-well culture plate (Corning, Corning NY) and incubated with 2 ml of medium containing $Na_2[^{35}S]O_4$ (50 μCi/ml, 12 h) to label newly synthesized proteoglycans. The volume and design of the loading chamber precluded labeling during the loading period. To label newly synthesized TGF-β the segments were incubated in medium containing Pro-Mix, a mixture of ^{35}S-methionine and ^{35}S-cysteine (4:1 ratio, 0.25 mCi/ml, 12 h, sp act >1000 Ci/mmol; Dupont NEN). After radiolabeling, the tendon segments were rinsed with Puck's saline three times for 20 min each, lyophilized, and weighed. The dry tissue weight of one segment was between 50 and 70 mg. Dry tissue was extracted at 25 mg dry wt of tissue per milliliter of extractant with guanidine buffer containing protease inhibitors (4 M guanidine–HCl, 50 mM sodium acetate, pH 6.0, with 2 mM EDTA, 5 mM benzamidine, 5 mM *N*-ethylmaleimide, 1 mM PMSF) at 4°C for 24 h. This single extraction step removes 80% of the ^{35}S-labeled macromolecules, of which 90% can be digested by chondroitin ABC lyase (9).

Immunoprecipitation of radiolabeled TGF-β. Components present in the 4 M guanidine extracts (0.5 ml of extract, representing 12.5 mg tissue) were processed into an immunoprecipitation (IP) buffer by first dialyzing the extracts of the tissue twice for 30 min in 1000 vol of 4 mM HCl and then overnight in 250 vol of 4 mM HCl containing 1 μg/ml of Pepstatin A and Leupeptin. A significant amount of material became insoluble during the dialysis step. The dialyzed samples were frozen, lyophilized, and then resuspended in 400 μl of IP buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% Na deoxycholate (Boehringer-Mannheim, Indianapolis, IN), 0.1% SDS, 0.005% merthiolate) and heated to 100°C for 5 min. Samples were precleared with 100 μg of a nonspecific rabbit IgG for 30 min at room temperature, utilizing either a 10% suspension (v/v) of formalin fixed *Staphlococcus aureus* cells (Staph A; Boehringer Mannheim, Indianapolis, IN) or a 10% suspension (v/v) of protein A–Sepharose as a source of protein A for collection of immune complexes. TGF-β was immunoprecipitated from the precleared samples with 60 μg of Marjan antibody and protein A-Sepharose (overnight, 4°C). Marjan is a purified rabbit polyclonal antibody specific for TGF-β1, β2, and β3 isoforms (25). The immunoprecipitates were washed four times in 1 ml of IP buffer and then extracted in 50 μl of gel sample buffer with heating for 5 min at 100°C. The entire immunoprecipitate from each sample was electrophoresed on a 12% SDS polyacrylamide gel and autoradiography performed using Kodak X-OMAT AR film.

It was possible to immunoprecipitate added ^{125}I-labeled TGF-β (NEN Dupont, Wilmington, DE) from tendon tissue extracts, but the efficiency of this step was lower than that for immunoprecipitation from the buffer alone (Fig. 1). Quantitation of each step of the procedure showed that 20% of the added ^{125}I-labeled TGF-β was lost during processing (dialysis and lyophilization) and an additional 10% during the nonspecific preclearing step; 60% of the ^{125}I-labeled TGF-β remaining after the preclear step was subsequently brought down with specific antiserum. Thus, a single immunoprecipitation procedure recovered 42% of the ^{125}I-labeled TGF-β added to an amount of tissue extract representing 12.5 mg dry wt of fetal tendon. This recovery was not changed when twice the amount of Marjan Ab was used, indicating that the specific antibody was present in a saturating amount. When Marjan antibody was omitted during the final precipitation only 8% of the added ^{125}I-labeled TGF-β was recovered in the pellet. These results demonstrate that a significant and repeatable percentage of TGF-β can be immunoprecipitated from an extract of fetal tendon tissue by this protocol.

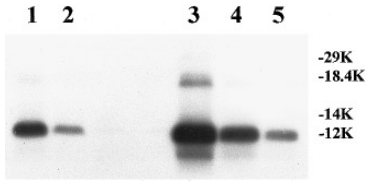

FIG. 1. Autoradiography of ^{125}I-labeled TGF-β after immunoprecipitation in the presence or absence of tissue components. Two picomoles of ^{125}I-labeled TGF-β was added to immunoprecipitation buffer or to guanidine extract of fetal tendon, which was then processed into immunoprecipitation buffer. Both samples were precleared with nonspecific rabbit IgG and Staph A membranes. TGF-β was then immunoprecipitated and samples analyzed under reducing conditions by 12% SDS/polyacrylamide gel electrophoresis. Lane 1, immunoprecipitaton from IP buffer; lane 2, immunoprecipitation from tendon tissue extract. Lanes 3, 4, and 5 show 2.0, 0.67, and 0.22 pmoles of ^{125}I-labeled TGF-β, respectively.

Immunoprecipitates from metabolically labeled control and loaded tendon segments contained a band with an apparent molecular weight after reduction like that of reduced ^{125}I-labeled TGF-β1 (12 kDa). The 12-kDa bands were absent in nonreduced samples, as would be expected for the disulfide-linked TGF-β dimer. Higher molecular weight radiolabeled bands were also present in the immunoprecipitates. When Marjan antibody was not present during the immunoprecipitation step, the signal for the 12-kDa bands was decreased more than 10-fold relative to signal for two irrelevant bands. The observation that a 12-kDa band was present after immunoprecipitation with Marjan, was diminished when Marjan was omitted, and was absent from nonreduced samples argues strongly that the 12-kDa band represents radiolabeled TGF-β. Unfortunately, the presence after reduction of a contaminating band migrating at ~24 kDa made it impossible to state that the 12-kDa band believed to represent TGF-β was migrating at 24 kDa when not reduced. The amount of radioactivity recovered in the immunoprecipitation pellet from tissue that was metabolically labeled after loading was only 0.02% of the total macromolecular radioactivity in the tissue extract.

Quantitation of the relative signal intensities for TGF-β was accomplished by digitizing the films with the aid of the Image-1 computer software package (Universal Imaging Co., Westchester, PA). Optical density values for TGF-β were determined by subtracting an average nonspecific background optical density, measured in regions just above and below each of the TGF-β bands, from the specific optical density for each TGF-β band. Attempts to detect TGF-β immunoprecipitated from extracts of unlabeled fetal tendon on Western blots were not successful, even when an ^{125}I-labeled secondary antibody was used. This is probably because there is so little present in the tissue.

Imunohistochemistry. Segments of fetal tendon and tissue from the fibrocartilaginous region of adult tendon were fixed overnight in cold 60% (v/v) buffered acetone, dehydrated through a graded series of ethanol, and embedded in paraffin. Sections (14 μm) were stained with an affinity-purified polyclonal antibody raised against a peptide corresponding to amino acid residues 328 to 353 of human TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA). An avidin–biotin–peroxidase detection system (Vector Laboratories, Burlingame, CA) was used to detect bound antibody.

RESULTS

Proteoglycan and Collagen mRNA Expression Following Mechanical Load

Segments of deep flexor tendons from the hind legs of near-term bovine fetuses were cultured for an initial 3-day period and then subjected to 72 h of cyclic loading. Northern blot (Fig. 2A) and slot-blot analysis (Table I) was performed on total RNA prepared from cells enzymatically liberated from both control segments and segments subjected to loading. Following loading the level of aggrecan mRNA was increased as much as 450%, the level of biglycan mRNA was increased 100%, and the level of versican mRNA was increased 130%, compared to nonloaded control segments. The level of decorin mRNA changed little, while the level of αl(I) collagen mRNA increased 40%.

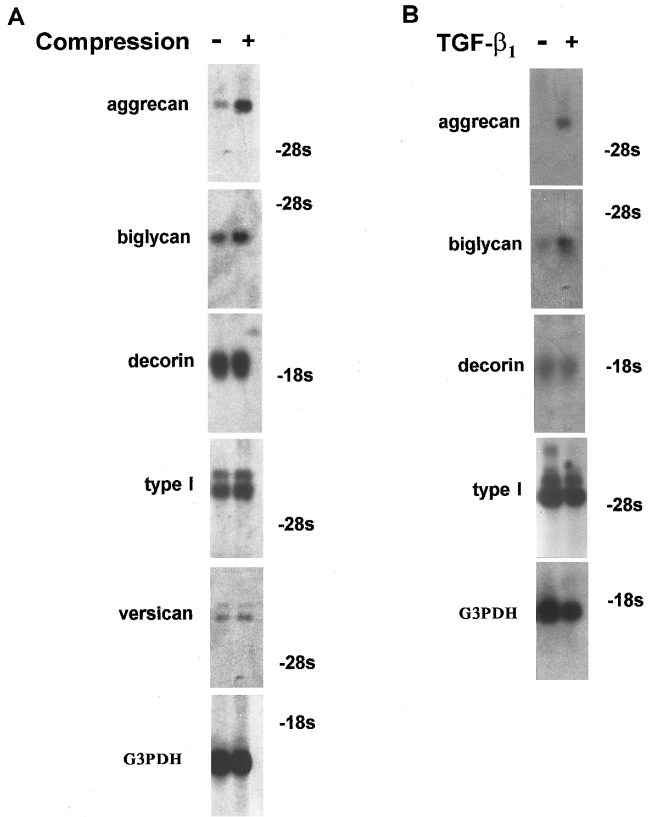

FIG. 2. Expression of mRNA for extracellular matrix molecules by fetal tendon treated with cyclic mechanical loading or TGF-β. Segments of fetal tendon were (A) cultured for 72 h with (+) or without (−) cyclic mechanical loading or (B) cultured for 72 h in medium that was (+) or was not (−) supplemented with TGF-β1 at 1 ng/ml, as described under Materials and Methods. Northern blot analysis was performed using ^{32}P-radiolabeled cDNA probes for aggrecan, decorin, biglycan, versican, and α1(I) collagen. Blots were also hybridized with a glyceraldehyde-3-phosphate dehydrogenase (G3PDH) probe. The position of 18S or 28S ribosomal RNA is indicated to the right of each panel.

TABLE I

Synthesis and mRNA Expression in Tendon Subjected to Cyclic Mechanical Loading or Cultured with Added TGF-β

	Cyclic loading		Added TGF-β
	24 h	72 h	(72 h)
Proteoglycan synthesis			
Aggrecan	N.D.	+200[a]	+175
Biglycan	N.D.	+110[a]	+250
Decorin	N.D.	+3[a]	+4
TGF-β synthesis	+/−	+310	N.D.
mRNA level			
Aggrecan	+50, +50[b]	+200, +450	+100, +140
Biglycan	N.D.	+80, +100	+120, +140
Decorin	N.D.	+20	−10
Versican	N.D.	+130	N.D.
α1(I) collagen	N.D.	+40	+40
TGF-β	+4	+40	+40

Note. Numbers represent percentage change in treated as compared to matched untreated tissue. Proteoglycan synthesis was determined after [^{35}S]sulfate incorporation, TGF-β synthesis after [^{35}S]methionine/cysteine incorporation and immunoprecipitation, and mRNA levels were determined by slot-blot hybridization, as described under Materials and Methods. N.D., not determined. +/−, insufficient signal intensity for accurate quantitation.

[a] Evanko and Vogel (9).

[b] Multiple values represent determinations made in separate experiments.

Proteoglycan Synthesis and mRNA Expression Following TGF-β Treatment

All tendon segments cultured in the presence of added TGF-β1 showed increased [^{35}S]sulfate incorporation into large proteoglycan and biglycan when compared to segments cultured without the added cytokine (Fig. 3). In this report, "large proteoglycan" synthesized by fetal tendon refers to ^{35}S-labeled macromolecules that elute in the V_o upon Sepharose CL-4B chromatography, migrate slowly on SDS/PAGE, and are >90% digested by chondroitin ABC lyase (26). It is likely that newly synthesized aggrecan and versican are both present in this fraction. The level of TGF-β-stimulated incorporation into large proteoglycan and biglycan was increased to a greater extent in the presence of NCS. For example, TGF-β stimulated only a 9% increase in

radiosulfate incorporation into large proteoglycan in tissue cultured in the absence of serum, compared to a 175% increase in tissue cultured in medium containing either 1 or 10% NCS. Similarly, sulfate incorporation into biglycan was increased 130% by TGF-β in the absence of serum and 250% in the presence of serum. This result also demonstrates that there was no significant level of active TGF-β in the serum. In contrast to the increased biglycan synthesis, sulfate incorporation into decorin remained largely unaffected or was even slightly diminished by added TGF-β.

To examine the effects of TGF-β on extracellular matrix gene expression, segments were cultured in 10% NCS with 1 ng/ml of TGF-β for 72 h and Northern blot analysis was performed (Fig 2B). Slot-blot analysis was utilized to quantitate changes in mRNA levels. TGF-β stimulated a >100% increase in the level of both aggrecan and biglycan mRNA as compared to segments cultured without added TGF-β (Table I). Following TGF-β treatment, decorin mRNA showed a slight decrease and αl(I) collagen mRNA was increased 40%.

These measurements, as summarized in Table I, demonstrate that the changes in proteoglycan synthesis and mRNA expression following TGF-β1 treatment were similar in pattern and direction to the changes in proteoglycan synthesis (9) and mRNA expression induced by mechanical load. The similarities suggested that TGF-β could be involved in the regulation of proteoglycan synthesis by mechanical load.

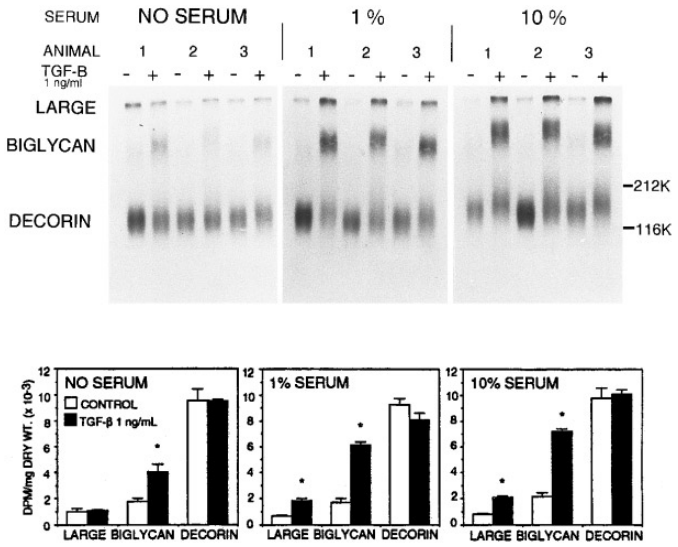

FIG. 3. Proteoglycan synthesis in segments of fetal tendon cultured in the presence of TGF-β. Matched tendon segments were cultured for 72 h in medium containing no serum, 1% serum, or 10% serum, with or without added TGF-β (1 ng/ml). During the final 12 h of the culture period [^{35}S]sulfate was added. Extracted proteoglycans were separated by 5.5–20% gradient SDS/polyacrylamide gel electrophoresis and visualized by autoradiography (top). Molecular weight standards were myosin (212 kDa) and β-galactosidase (116 kDa). To quantitate each type of proteoglycan a replicate gel was cut into strips representing each lane, divided into 5-mm sections, and counted (bottom). Bars indicate SEM, $n = 3$ segment pairs; asterisks indicate significant increase ($P < 0.05$) using a paired t test.

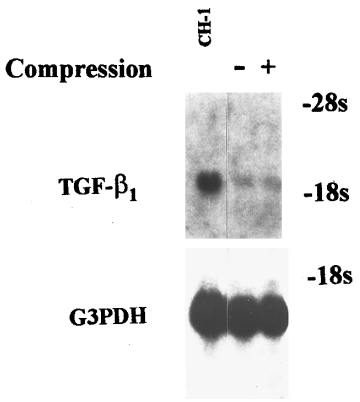

FIG. 4. Expression of TGF-β mRNA. Northern blots were prepared with 8 μg of total RNA from a human chondrosarcoma cell line that expresses abundant TGF-β mRNA (CH-1) and from fetal tendon segments cultured for 72 h without (−) or with (+) cyclic mechanical load. The blot was hybridized with ^{32}P-labeled cDNA probes for TGF-β1 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The positions of 18S and 28S ribosomal RNAs are indicated to the right of each panel.

TGF-β1 mRNA Expression Following Mechanical Loading and TGF-β Treatment

The level of TGF-β1 mRNA expressed by cells in cultured fetal bovine tendon segments was quite low (Fig. 4). RNA prepared from a human chondrosarcoma cell line (27) was included in the analysis as a positive control. Slot-blot analysis was performed to quantitate changes in TGF-β1 mRNA expression following mechanical loading. No change in the level of TGF-β1 mRNA was seen following 24 h loading. However, a 40% increase in the level of TGF-β1 mRNA was measured following 72 h loading, compared to matched segments that were not loaded. Northern blots and slot blots were also hybridized with probes specific for TGF-β2 and TGF-β3; however, no signal for these transcripts was observed in either the control or loaded samples, even after 3 weeks of exposure (data not shown).

Total RNA was also prepared from segments incubated for 72 h in medium supplemented with 10% serum with or without added TGF-β at 1 ng/ml, and analyzed for TGF-β mRNA expression by slot blot analysis. TGF-β treatment increased TGF-β mRNA expression by approximately 40%.

Quantitation of TGF-β Synthesis after Loading

A greater amount of newly synthesized TGF-β was immunoprecipitated from tendon segments subjected

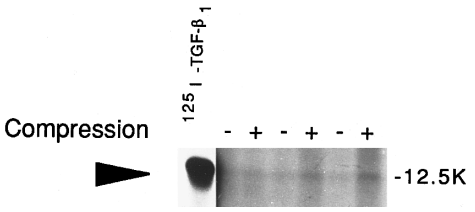

FIG. 5. Immunoprecipitation of ^{35}S-labeled TGF-β synthesized by tendon segments subjected to cyclic mechanical load. Tendon segments cultured for 72 h with (+) or without (−) mechanical load were radiolabeled with [^{35}S]methionine and [^{35}S]cysteine (4:1 ratio) for 12 h immediately following the loading regimen. TGF-β was immunoprecipitated from tissue extract, reduced, and analyzed by 12% SDS/polyacrylamide gel electrophoresis and autoradiography, as described under Materials and Methods. The film was exposed for 14 days. Each lane contains the total immunoprecipitate from extract representing 12.5 mg dry wt of tissue. Reduced ^{125}I-labeled TGF-β was loaded on the same gel; it was readily located after only 1 day of exposure (lane 1).

to cyclic load for 72 h than from matched unloaded segments (Fig. 5). The intensity of the 12-kDa band increased 310% ($P < 0.05$, paired-sample t test; $n = 4$) in the loaded segments as compared to controls. TGF-β was also immunoprecipitated from tendon segments loaded for only 24 hr and analyzed in a similar manner. After 24 h loading, the signal for TGF-β was even more faint than that after 72 h (data not shown), and no difference between control and loaded samples could be measured.

Immunolocalization of TGF-β1

Sections of freshly isolated fetal tendon, cultured control and loaded fetal tendon, and fibrocartilage from adult tendon were stained with antibody specific for TGF-β1. The staining observed in fresh fetal tendon (tissue which had not been cultured) was barely more than the negative controls (Fig. 6A). Both the loaded and unloaded tissue which had been in culture for a total of 6 days showed cellular staining and slight positive extracellular staining with the TGF-β1 Ab (not shown). However, there was no noticeable difference in the distribution or intensity of the staining in control vs loaded tissue. In the fibrocartilaginous region of adult tendon intense intracellular staining was observed in the cartilage-like cells (Fig. 6B).

DISCUSSION

Fibrocartilage develops in tendons at their entheses and at midsubstance sites where the tissue is subjected to transverse compressive loading in addition to tension (1, 3, 7, 28–30). A key characteristic of the fibrocartilage is accumulation of large proteoglycan (aggrecan) which gives the tissue the ability to withstand compressive load. The midsubstance transition from tendon to fibrocartilage is primarily a postnatal event in the bovine deep flexor tendon, with the tissue being fully developed by ~6 months of age (26). The *in vitro* experiments of this study were designed to mimic several aspects of conditions under which the tendon-to-fibrocartilage transition occurs *in vivo*, in order to investigate the mechanisms involved. We conclude that both mechanical load and the cytokine TGF-β may be involved in this transition, based on the observations that (i) both mechanical loading and TGF-β stimulated a similar pattern of specific changes in proteoglycan synthesis and gene expression, and (ii) mechanical loading stimulated increased TGF-β synthesis.

Previous experiments showed that application of cyclic, uniaxial compressive load to segments of fetal bovine tendon stimulated increased synthesis of large proteoglycan and biglycan (9). The current study confirms and extends these observations and also shows that adding TGF-β1 to the medium in which fetal ten-

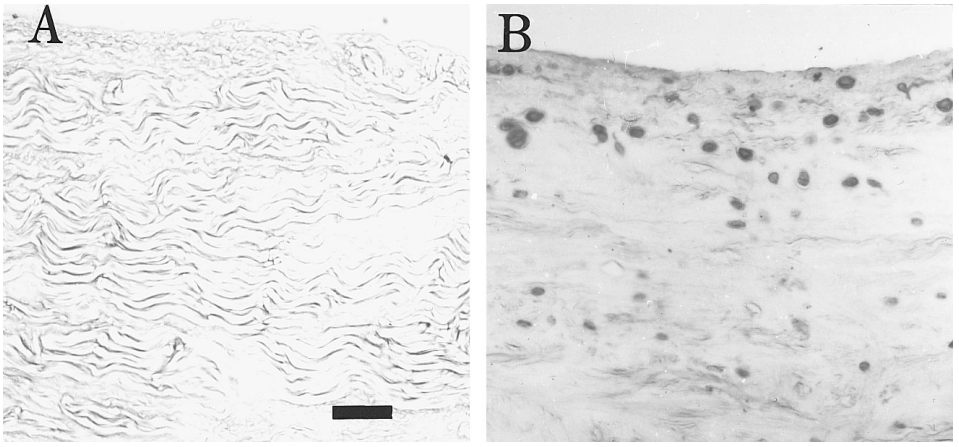

FIG. 6. Immunolocalization of TGF-β in fetal and adult tendon. Paraffin sections from a freshly harvested fetal tendon (A) and from the compressed region of adult tendon (B) were stained with a polyclonal antibody specific for TGF-β1. Very little staining for TGF-β was observed in fresh fetal tendon. In adult tissue the round cells close to the compressed surface of the tendon were intensely stained for TGF-β. Bar, 50 μm.

don segments are cultured affects proteoglycan synthesis in a manner that is remarkably similar to the changes induced by cyclic compressive load. The characteristics shared by both treatments include increased mRNA expression and synthesis of aggrecan and biglycan, increased glycosaminoglycan chain length, and increased C6S/C4S sulfation ratio (9, 31, 32). There was little or no change in decorin mRNA expression or synthesis or expression of type I collagen mRNA. The specificity of this cellular response to TGF-β has been noted by other investigators (33) and is further supported by the observation that addition of TGF-β_1 (1 ng/ml) to monolayer cultures of bovine tendon fibroblasts induced a specific increase in large proteoglycan and biglycan synthesis in the presence of 1% or 10% newborn calf serum, whereas the addition of platelet derived growth factor (100 ng/ml) or insulin-like growth factor-1 (10 ng/ml) induced increased synthesis of only the large proteoglycans, and only when cultures were in 1% serum (31, and K. Vogel, unpublished observation).

Similarity in the cellular responses to compressive loading and TGF-β treatment suggested that compressive loading may increase the amount of active TGF-β in the tissue, and this activity could subsequently induce increased expression and synthesis of aggrecan and biglycan. We measured increased synthesis of radiolabeled TGF-β and slightly increased levels of TGF-β1 mRNA expression in tendon explants subjected to 72 h *in vitro* loading. Neither of these changes was detected following 24 h loading. Furthermore, aggrecan synthesis and mRNA expression was increased more after 72 h of loading than after 24 h loading (see also 9). This delayed response is consistent with changes in proteoglycan synthesis following the development of a more immediate response, such as increased cytokine activity.

Previous studies have demonstrated that mechanical loading can stimulate a rapid increase in TGF-β activity. A 3- to 5-fold increase was measured in the medium of aortic endothelial cell cultures subjected to fluid shear stress for 2 h (18), whereas an increase $>$10-fold was measured in the medium of periosteal cells stimulated by intermittent hydrostatic pressure for 3 days (17). In these studies a cell proliferation inhibition assay was used to measure TGF-β activity in the cell culture medium. Unfortunately, it was impossible to use a cell-proliferation assay to measure a change in the amount of active TGF-β in our experiments because guanidine extraction activates latent TGF-β (34). All attempts to measure TGF-β activity released from tendon to the culture medium were negative (Evanko and Vogel, unpublished observation). The level of mRNA for TGF-β was reported to be 2-fold higher within 4 h of brief *in vivo* mechanical loading of rat tibial periosteum (35) and as much as 5-fold higher in cultured endothelial cells exposed to high shear stress for 4 h (18). These changes are significantly greater and more rapid than we found in the loaded bovine tendon explants. At this point we cannot know whether this is because of inherent differences in response between tendon and bone cells, or whether the load that we imposed on tendon (a complex load with both shear and normal stress components) was a less effective stimulus. None of the earlier studies measured synthesis of TGF-β.

The experiments reported here have not allowed us to distinguish between alternative mechanisms through which the amount of active TGF-β could be increased within the tendon tissue. One possibility is that loading directly stimulated cells to increase expression and synthesis of TGF-β. Alternatively, loading could have stimulated release and/or activation of TGF-β stored in the tendon ECM, perhaps through cleavage of a latent TGF-β binding protein (36). This increased amount of active TGF-β could then stimulate increased synthesis of the cytokine, a response that has been previously noted (37) and is consistent with our data. Our results suggest that TGF-β synthesis was not directly correlated with increased mRNA expression. Change in TGF-β synthesis without a corresponding change in mRNA level, i.e., posttranscriptional regulation, has been reported before in cell culture systems (37, 38). For example, TGF-β mRNA was expressed at similar levels in stimulated and unstimulated monocytes in culture. However, the cytokine was only secreted by stimulated macrophages (38).

Few investigators have attempted to quantitate newly synthesized TGF-β in connective tissue, and we do not know of any other attempts to compare levels of synthesis following a particular treatment. This experiment was attempted because other approaches had obvious problems. For example, calf cartilage contains high levels of stored inactive TGF-β (39), and this may have been true for tendon as well. Using an ELISA assay to measure the amount of total TGF-β in tendon would not be useful if loading induced only a small change in the overall amount. Adding anti-TGF-β antibodies to cultured explants to block cytokine activity during loading was also considered. However, large quantities of antibody were not available and a negative result would not have been definitive because neither antibody penetration nor the site of active cytokine was known. We were concerned that immunoprecipitation of newly synthesized TGF-β may have been prevented by a high level of nonlabeled growth factor present in the tissue extract. In fact, this turned out not to be a serious problem, perhaps because, as indicated by immunohistochemistry, there is little TGF-β stored in uninjured normal tendon extracellular matrix (40, 41). Even in the presence of extracted tendon tissue $\sim$40% of the added ^{125}I-labeled TGF-β1 was regularly immunoprecipitated. This is the basis upon which quantitative immunoprecipitation was undertaken. It is recog-

nized, of course, that the quantitation assumes immunoprecipitation with equivalent efficiency from all samples. The greatest problem of this experimental approach was that more than 99.98% of the newly synthesized proteins labeled during our protocol were *not* TGF-β. Even with specific antibodies the amount of background radioactivity was higher than the specific signal due to TGF-β. For this reason, changes in newly synthesized TGF-β could only be approximated by densitometry of the TGF-β bands after gel electrophoresis and autoradiography.

Although the vast majority of studies investigating the regulation of extracellular matrix synthesis by cytokines have utilized cells in culture, it is never clear whether the response of cells removed from their usual extracellular matrix are a valid indication of events in the tissue from which those cells were derived. Studies which attempt to understand transitions in connective tissue structure are inherently difficult because of the complexity of the tissues and the extended time required for changes in tissue composition and organization to take place. The development of fibrocartilage in tendon provides an excellent model to begin testing tissue-level mechanisms which regulate the development of specific tissue composition and structure. The *in vitro* model is of interest because the response of fetal tendon cells in tissue to applied mechanical loading includes changes that involve the same molecules and are in the same direction as changes that occur over a longer time *in situ*. It is not yet clear whether a small change in TGF-β expression can regulate ECM composition. In addition, we recognize that the direct stimulus for changed expression of matrix genes could be a factor stimulated by TGF-β rather than TGF-β itself (42). The experiments of this study support an hypothesis suggesting that cells in fetal tendon respond to compressive load with cytokine changes that, in turn, stimulate synthesis of extracellular matrix molecules and lead toward appropriate fibrocartilage formation.

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