Original Article

In vitro culture decreases the expression of TGFβ, Hsp47 and type I procollagen and increases the expression of CTGF in avian tendon explants

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*This paper is dedicated to the memory of Dr. George N. Rowland III

Abstract

Weight-bearing tendons in many species, including humans, chickens and horses, are prone to failure, in many cases without a discernible cause. The normal function of the tendon depends on the proper assembly of fibrils of type I collagen, the main structural component of the tendon. We studied the effect of *in vitro* culture, temperature (37ºC vs. 43ºC) and wounding on the expression of mRNAs for several collagen regulators, transforming growth factor beta (TGFβ), heat shock protein 47 (Hsp47) and connective tissue growth factor (CTGF), in chicken embryonic gastrocnemius tendon explants. The expression of mRNAs for TGFβ and Hsp47, a chaperone of collagen assembly, remained strong during the first day of *in vitro* culture, but then it decreased, slightly more at higher temperature. Additional injury in selected tendons had no significant effect on the levels of TGFβ and Hsp47 mRNAs. Likewise, the level of immunostained type I procollagen also decreased with the length of culture. The expression of CTGF gradually increased from 0 at the time of tendon removal with the duration of culture to strong after three days of culture when the expression of TGFβ and Hsp47 was low. We conclude that *in vitro* culture over the period of several days rather than an increase in temperature or additional wounding decreases the expression of TGFβ, Hsp47 and type I procollagen and increases the expression of CTGF.

Keywords: Tendon Explants, *In Vitro* Culture, TGFβ, Hsp47, CTGF, Type I Procollagen

Introduction

Tendons and fibrillar collagen, their main component, undergo a significant degree of remodeling associated with movement and loading. Stress/injury and posttraumatic repair are first characterized by degradation of injured tissue before its replacement and remodeling. The majority (95%) of fibrillar collagen in the tendon is type I that provides tensile strength and is organized into fibrils (at the electron microscopic level) and fibers (at the light microscopic level). Type III collagen, present in much smaller quantities, contributes to tissue expansibility, attachment of tendon to periosteum and proper healing after injury[1]. Our understanding of regulation of collagen production and structure in normal and stressed tendon is rather limited. We know that several proteins regulate the production and proper assembly of collagen. However, the relationship between stress and injury and the trigger to collagen remodeling is less clear. Synthesis of collagen is directly stimulated by transforming growth factor beta (TGFβ)[2,3]. Elevated TGFβ levels have been demonstrated in a wide variety of fibrotic disorders in humans and other species, and decreased TGFβ activity has been associated with impaired healing.

Connective tissue growth factor (CTGF) is a mitogenic and chemotactic heparin-binding protein that is secreted by fibroblasts after activation by TGFβ. CTGF is a member of

a highly conserved family of peptides related to PDGF. This group includes the murine genes *cyr 61* and *fisp 12*, *xnov* in Xenopus, and a Drosophila gene, *tsg*[4,5]. Closely related avian genes, *cCTGF* and *cef-10*, have also been identified (GenBank Accession AJ298335)[6]. Amino acid sequence identity to human CTGF is over 90% in the murine homologue. High sequence homology is found with the more closely related *cCTGF* (GenBank Accession AJ298335). Co-ordinated expression of CTGF and TGFβ has been demonstrated in both mammalian models of normal wound repair and in several fibrotic diseases in animals and humans[7,8]. CTGF is tightly regulated by TGFβ and previous studies have suggested that many of the effects of TGFβ on connective tissue formation, including type I collagen induction and fibroblast proliferation, are mediated by CTGF rather than PDGF, fibroblast growth factor or epidermal growth factor[7,9,10].

Other proteins are less direct regulators of collagen synthesis. One of them, heat shock protein 47 (Hsp47), is a member of the family of molecular chaperones that enable proper folding and assembly of other proteins. They are upregulated during stress, such as elevated temperature when rapid mobilization of already synthesized but not yet assembled proteins is necessary. Hsp47 is a heat shock protein with M_r 47,000 that serves as a collagen-binding chaperone[11]. Hsp47, a member of the serpin family (plasma serine protease inhibitors), is located in the endoplasmic reticulum where it directs proper folding and elongation of nascent α1(I) procollagen chains[12]. Hsp47 likely promotes stability in the triple-helical region of procollagen, especially during heat stress when Hsp47 binding would prevent microunfolding[13].

Little is known about regulation of tendon collagen synthesis in types of stress other than mechanical injury. It is known that young broiler chickens are prone to failure of the gastrocnemius tendon, especially during hot humid summer months experienced in the southern United States[14]. It is not clear whether human gastrocnemius (Achilles) tendon that is prone to injuries undergoes stressful changes associated with overheating during intense exercise and whether the effect of temperature induces direct changes in the tendons (e.g., tendon shrinkage and degeneration of collagen fibers) or whether temperature modulates the expression of some of the regulatory elements just described. Vangsness et al. have shown that application of heat, in the form of laser energy, induces tendon shrinkage (10% of the tendon length) and biomechanical impairment likely due to denaturation and degeneration of collagen fibers[15]. Hyperthermia has been demonstrated *in vivo* in the central core of equine superficial digital flexor tendons during gallop exercise. The temperature in the core has been recorded as high as 45°C, while the temperature at the surface was 5°C less[16]. Though tendon fibroblasts are fairly resistant to hyperthermia, repeated exposure of these cells to high temperature decreases their survival *in vitro*, and may result in tendon central core degeneration *in vivo*[17]. The central core of the tendon, i.e., the site of temperature increases, is also the site

of degeneration and subsequent injury in the equine flexor tendon[18] and human Achilles tendon[19]. We hypothesized that elevated environmental temperature may lead to an increase of the expression of genes regulating synthesis of type I collagen in the tendon. To test our hypothesis we examined the level of expression of mRNAs for TGFβ, Hsp47 and CTGF and on the presence of immunostained type I procollagen in avian embryonal tendon explants maintained at 37°C and 43°C up to 1 week. We selected 37°C as the "normal" temperature for *in vitro* maintenance of chicken explant because most likely the temperature of a superficially located tendon, as is the gastrocnemius, is lower than the internal body temperature. The induction of Hsp47 is usually studied at 43°C or 45°C[11,20]. We report that the length of *in vitro* culture rather than increased temperature or additional injury had a significant effect on expression of TGFβ, Hsp47, CTGF and type I procollagen.

Materials and methods

Tendon explant cultures

Institutional Review Board Approval was obtained before the use of chicken embryos. Gastrocnemius tendons from 18-day-old broiler chicken embryos were aseptically dissected from bone and muscle. Seven to ten mm long tendon segments were removed with the sheath attached. Some tendons were immediately snap frozen. All other tendons were placed into 35 mm culture dishes with Dulbecco's modified Eagle medium (DMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS). In addition, one half of the tendons were cut transversely at 3-4 mm intervals through the tendon sheath and approximately 1/3-1/2 into the tendon proper. These tendons are called "wounded" in the text. Tendons were incubated at 37°C or 43°C in a humidified atmosphere of 5% CO_2 and 95% air from time 0 for up to a week. Preliminary experiments determined that the temperature of 43° was well tolerated by the explants (the normal core temperature of chicken is 41°C). These temperatures were selected to test the effect of below-normal and above-normal temperatures on chicken tendon. The explant cultures appeared fully viable even after a week of *in vitro* maintenance and the culture medium maintained neutral pH. Upon removal from the medium, tendons were immediately frozen at -70°C. The experiments were repeated four times, with 2-3 tendon explants harvested per data point (from 1 hr up to 1 week) in each experiment.

Northern blot analysis

Short-term tendon cell cultures were established from 18-day-old chicken embryonal gastrocnemius tendons following a modified method of Kempka et al.[21,22]. Aseptically dissected gastrocnemius tendons were stripped of the tendon sheath and cut under sterile conditions with scissors into 1-2 mm long pieces. No further separation of tendon cell types

was attempted. The pieces were digested with 3 mg type I or type II collagenase/ml DMEM at 37°C for 12 to 18 hours. The cell suspension was filtered through a 70 μm nylon mesh, washed in phosphate-buffered saline (PBS) and was suspended in DMEM supplemented with 13% FBS. The tendon cells were grown in 60 mm tissue culture dishes in DMEM with 10% FBS. Confluent monolayers were scraped off with a sterile plastic cell scraper and the collected cells were used to extract total RNA with TRIZOL reagent (Life Technologies, Inc., Grand Island, NY). The extraction is based on the guanidium thiocyanate and chloroform/isoamyl alcohol extraction method of Chomczynski and Sacchi[23]. Total RNA was denatured at 65°C for 10 minutes, separated in 1.4% formaldehyde/agarose gel (3-10 μg RNA per lane), transferred to nylon membrane and hybridized with a digoxigenin-labeled TGFß or Hsp47 probes. Both prehybridization and hybridization were executed at 42°C for 2 hours and overnight, respectively, using the same hybridization buffer as described for ISH below. After hybridization, membranes were washed with 2 X SSC, 0.5% SDS for 15 minutes at room temperature (RT), then with 0.1 x SSC, 0.1% SDS for 2 x 15 minutes at 42°C, and finally with 0.1 X SSC, 0.1% SDS for 2 x 15 minutes at 68°C. Digoxigenin detection was performed as described for ISH. Digoxigenin-labeled RNA molecular markers were obtained from Roche Boehringer Mannheim (Indianapolis, IN).

In situ hybridization assay for TGFβ

We constructed a 512 bp long RNA probe complementary to a segment of 2745 bp long human TGFβ1 mRNA between 1486 and 1998 bases. This probe has 92% homology to the corresponding chicken TGFβ1 cDNA sequence and 73% homology to chicken TGFβ3 cDNA sequence[24]. We used the following set of primers:

forward primer:
5'-<u>ATTTAGGTGACACTATAT</u>ATGAGGGCTTTCGCCTTAG-3'

reverse primer:
5'-<u>TAATACGACTCACTATAGGG</u>CGCACGCACGAT CATGTTGGAC-3'. The standard SP6 and T7 RNA polymerase (underlined) promoters were incorporated into the forward and reverse primers, respectively. The total RNA was isolated from a human pituitary adenoma using the total RNA extraction kit from Promega Corporation (Madison, WI). Reverse transcription polymerase chain reaction (RT-PCR) was performed using the Titan One Tube RT-PCR System (Roche) with the extracted total RNA first reversely transcribed into cDNA. The cDNA was used to amplify the specific sequence of the cDNA determined by the primers in 35 cycles of PCR (denaturation at 94°C for 30 seconds, annealing at 57°C for 30 sec, extension at 68°C for 2 minutes). The identity of the amplified DNA band with TGFβ1 cDNA sequence was verified by DNA sequencing. As the sequences of human and chicken TGFβ are highly homologous[24-26] we used the human probe.

Labeled riboprobes for use in ISH were then synthesized by *in vitro* transcription of the amplified DNA template utilizing the digoxigenin labeling kit for RNA (Roche). The anti-sense probe containing the T7 promoter was used to identify TGFβ mRNA in tissue sections; a sense probe with SP6 promoter sequence served as a negative control. Seven to ten μm thick frozen sections cut from tendons were washed in diethyl pyrocarbonate (DEPC)-treated PBS, and treated with proteinase K (25 μg/ml) at 37°C for 15 minutes, and then in 0.25% acetic anhydride in 100 mM tri-ethanolamine (v/v) for 10 minutes to prevent non-specific binding of the probe. Prehybridization in buffer consisting of 5 X SSC, 5 X Denhardt's solution, 50% deionized formamide and 250 μg/ml yeast tRNA was done at 42°C for 1 hour. The same buffer containing 30 ng/slide of a digoxigenin-labeled probe was applied to sections for overnight hybridization at 42°C. The next morning the slides were washed in solutions of decreasing SSC concentration. The slides were then incubated in buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5), blocked in 2% normal sheep serum in buffer 1 and incubated with alkaline phosphatase conjugated to anti-digoxigenin mouse monoclonal antibody diluted 1:300 in buffer 1 with 1% normal sheep serum for 2 hours at RT. The slides were washed 3 x in buffer 1 and equilibrated in buffer 2 (100 mM Tris-HCl, 100 mM NaCl, pH 9.5). The sections were stained with NBT/BCIP solution for up to 1 hour, air-dried and coverslipped. The intensity of staining, always limited to the cytoplasm, was quantified and analyzed statistically (see below).

ISH assay for Hsp47 mRNA

A segment (300 bp) of Hsp47 cDNA sequence was amplified from RNA extracted from primary embryonal tendon cell cultures using RT-PCR and primers with incorporated SP6 and T7 polymerase promoters to facilitate transcription into riboprobes. The chicken cDNA sequence (GenBank accession no. X57157) was the basis for the primer design. The primers encode for a region between positions 1062 and 1323. This region is located in the endoplasmic reticulum, presumably near the collagen-binding site. We used the following set of primers:

forward primer:
5'-<u>ATTTAGGTGACACTATA</u>ATCCAACGTCTTCCATGCC-3'

reverse primer:
5'-<u>TAATACGACTCACTATAGGG</u>AATCCCCCCC TAAAAAACAC-3'

RT-PCR and ISH, including evaluation of staining, were performed as described for TGFβ ISH.

ISH assay for CTGF mRNA

The CTGF cDNA oligoprobe encompasses a 50 bp highly conserved sequence shared by murine *fisp 12 (mCTGF)* and human *CTGF*. This portion of the gene corresponds to the active site in this family of peptides and begins with the cystine at amino acid position 210 of CTGF. The sequence of the probe is
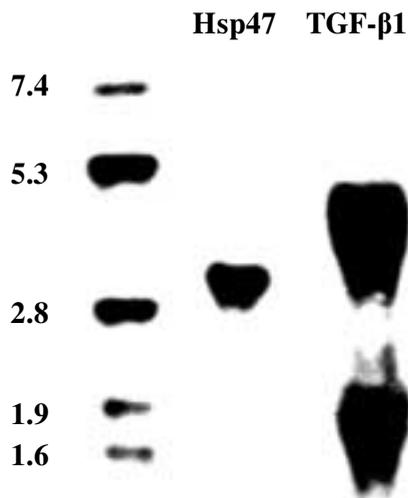
Hsp47   TGF-β1

7.4

5.3

2.8

1.9

1.6

Figure 1. Northern blot for Hsp47 and TGFβ mRNA. A composite picture of results from Northern blots on RNA extracted from chicken embryonal tendon cell cultures. Lane 1: RNA molecular markers. Lane 2: Hsp47 mRNA was expressed as a single band migrating at 3.3 kb. Lane 3: TGFβ mRNA migrated as two bands, one at 4.1 kb, the second at 1.8 kb.

5'-GTTGTCATTGGTAACCCGGGTGGAGATGCCT GTCCCACAGGTCTTGGAACA-3'. Frozen sections of tendons were prepared as for TGFβ and ISH performed similarly with the following modifications. After proteinase K digestion, slides were soaked in 4% paraformaldehyde for 20 min and then in PBS for 5 minutes. This was followed by a 30 s wash in 50% ethyl alcohol prior to acetylation. Oligoprobes were digoxigenin-labeled with an oligoprobe digoxigenin tailing kit according to manufacturer's directions (Roche). Hybridization was performed overnight at 40°C. Washes and digoxigenin detection were performed as described for TGFβ. The sections were counterstained with hematoxylin. The intensity of staining, always limited to the cytoplasm, was quantified and analyzed statistically (see below).

Immunohistochemistry

Monoclonal antibodies to type I procollagen and type III collagen were used to visualize respective fibrillar collagens[27,28]. An immunoperoxidase protocol used by us previously was employed[22]. Ten micron thick frozen sections were cut from previously frozen tendons. The sections were first air-dried for at least a day at RT. Upon washing in PBS, endogenous peroxidase activity was quenched in 0.03% H_2O_2(v/v) in H_2O for 30 minutes at RT. Nonspecific binding sites were blocked with 2% horse serum in PBS for 1 hour at RT. The mouse monoclonal antibodies to chicken type I procollagen (M-38) and type III collagen (3B2, both from Developmental Studies Hybridoma Bank maintained by The University of Iowa, Department of Biological Sciences, Iowa City, Iowa

52242, under contract NO1-HD-7-3263 from the NICHD) were used at dilutions 1:100 in PBS overnight at 4°C. An irrelevant primary antibody was used as a negative control. Secondary goat anti-mouse biotinylated antibody (1:1,000 in PBS) was applied for 1 hour at RT, followed by 1 hr incubation with ABC (avidin-biotin-complex) reagent (Vector Laboratories, Burlingame, CA) at RT. 3,3'-diaminobenzidine tetrachloride (DAB) served as a chromogen. The semiquantitative evaluation of intensity of staining, always limited to the cytoplasm, was analyzed statistically (see below).

Statistical analysis

Comparison of intensity of staining in *in situ* hybridization and immunohistochemistry assays among multiple groups was done using the general linear model algorithm of SAS (SAS Institute Inc., Cary, North Carolina) to determine significant effects of wounding, temperature, and duration of explant culture. No staining was evaluated as 0, the maximum score (3) was assigned to a section(s) with maximum staining in each group (TGFβ, Hsp47, CTGF or type I procollagen). This was performed under microscopic control by one of us (JH). Differences in the mean values of different groups were measured by analysis of variance followed by alternative *t* test, significance is reported as two sided *p* value. The experiments were repeated four times, with 2-3 sections per data point evaluated in each experiment.

Results

Expression of TGFβ and Hsp47 mRNAs in embryonal tendon cell cultures

Northern blotting revealed that Hsp47 mRNA was expressed as a single band migrating at 3.3 kb. The expression of TGFβ mRNA was characterized by two bands migrating at 4.1 and 1.8 kb (Figure 1). Based on the size, we propose that the 4.1 kb species is TGFβ2 mRNA and that the 1.8 kb band most likely represents mRNA for TGFβ4 isoform as these sizes were reported for these isoforms[24,26].

Regulation of TGFβ mRNA expression

In 18-day-old embryonal gastrocnemius tendons, the most significant changes in TGFβ mRNA expression occurred with the length of the incubation time of the explants. TGFβ mRNA was distributed evenly and with strong intensity in tendon and sheath fibroblasts immediately after excision and after 4 hr of explant culture at 37°C and 43°C (Figures 2B and C, Table 1). After 48 hours in culture a slight, statistically insignificant decrease in the intensity of expression in intact tendons kept at 37°C was observed (Figure 2D, Table 1). The staining was significantly decreased in wounded tendons maintained at 37°C and in both intact and wounded tendons kept at 43°C (Figure 2E, Table 1). With further incubation the level of TGFβ mRNA continued to be low
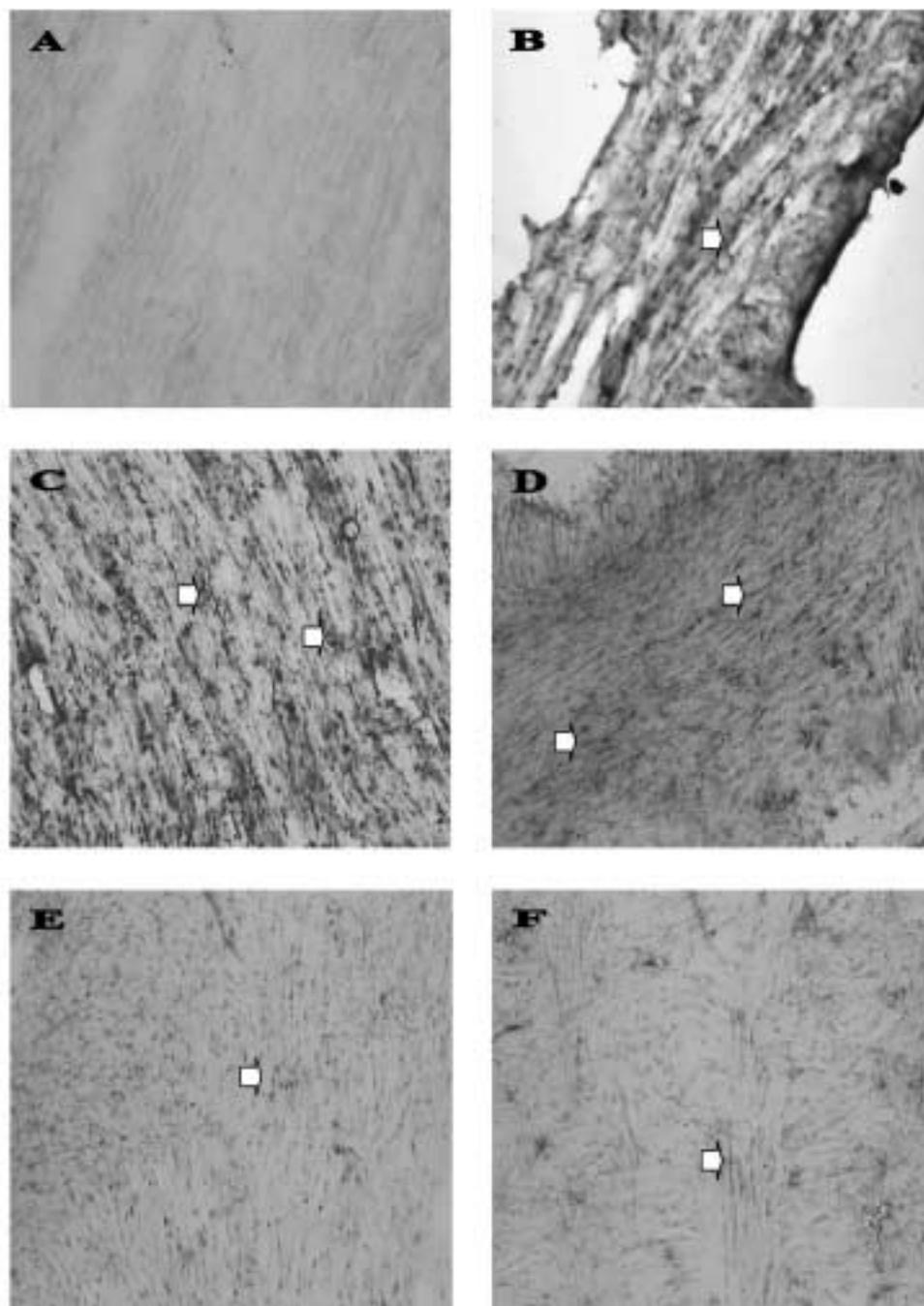
Figure 2. ISH for TGFβ mRNA. (A) Application of sense probe led only to faint background staining. ISH revealed strong cytoplasmic expression of TGFβ mRNA at the time of removal (B) and after 4 hours of culture in intact tendons kept in culture at 37°C (C). (D) After 48 hours in culture the expression of TGFβ mRNA was only slightly decreased in intact tendons at 37°C. Significant decrease in expression was observed in intact tendons kept at 43°C for 48 hours (E) and in intact tendons maintained at 37°C for 96 hr (F). Arrows point to specific staining for mRNA. Magnification x 400.

after 96 hours of culture (Figure 2F, Table 1). The explant cultures appeared fully viable even after a week of *in vitro* maintenance and the culture medium maintained neutral pH. No staining was observed when the antisense probe was replaced with a sense probe (Figure 2A).

Evaluation of expression of Hsp47 mRNA by ISH

The staining in tendons was mild to moderate immediately after excision. It increased, though to an insignificant degree, in intact and wounded tendons kept at 37°C and 43°C

Hr of incubation	Intact, 37°C	Wounded, 37°C	Intact, 43°C	Wounded, 43°C
0	2.6 ± 0.55[a]	2.6 ± 0.55[a]	2.6 ± 0.55[a]	2.6 ± 0.55[a]
4	2.61 ± 0.6[a]	2.55 ± 0.52[a]	2.17 ± 0.52[a]	2.47 ± 0.67[a]
48	1.125 ± 0.85[a]	0.786 ± 0.64[b]	0.75 ± 0.65[b]	1.0 ± 1.1[b]
96	0.8 ± 0.95[b]	1.1 ± 0.65[a]	2.5 ± 0[a]	0.7 ± 0.27[b]

Table 1. The differences in means of intensity of staining for TGFβ mRNA (± standard deviation) among different groups were measured by analysis of variance. The range of intensity was from 0 (no staining) to 3 (maximum staining). Dissimilar letters indicate significantly different groups at *P<0.05*.

Hr of incubation	Intact, 37°C	Wounded, 37°C	Intact, 43°C	Wounded, 43°C
0	1.5 ± 0.5[a]	1.5 ± 0.5[a]	1.5 ± 0.5[a]	1.5 ± 0.5[a]
1	3.0 ± 0[a]	2.25 ± 0.89[a]	1.0 ± 0[a]	1.71 ± 1[a]
12	2.0 ± 0[a,*]	2.67 ± 0.57[a,*]	1.5 ± 0.65[a,*]	1.33 ± 0.52[a,*]
48	1.33 ± 0.85[a,+]	1.1 ± 0.5[a,+]	1.0 ± 1[a,+]	0.625 ± 0.38[a,+]

Table 2. The differences in means of intensity of staining for Hsp47 mRNA (± standard deviation) among different groups were measured by analysis of variance. The range of intensity was from 0 (no staining) to 3 (maximum staining). Dissimilar letters indicate significantly different groups at *P<0.05*. * indicates statistically significant difference between combined treatments at 1 and 12 hours of incubation. + indicates statistically significant difference between combined treatments at 1 and 48 hours of incubation.

Hr of incubation	Intact, 37°C	Wounded, 37°C	Intact, 43°C	Wounded, 43°C
0	0 ± 0[a]	0 ± 0[a]	0 ± 0[a]	0 ± 0[a]
72	1.0 ± 0[b]	1.0 ± 0[b]	1.5 ± 0.7[b]	1.5 ± 0.7[b]
144	3.0 ± 0[c]	4.0 ± 0[c]	3.0 ± 0[c]	3.75 ± 0.5[c]

Table 3. The differences in means of intensity of staining for CTGF mRNA (± standard deviation) among different groups were measured by analysis of variance. The range of intensity was from 0 (no staining) to 3 (maximum staining). Dissimilar letters indicate significantly different groups at *P<0.05*.

Hr of incubation	Intact, 37°C	Wounded, 37°C	Intact, 43°C	Wounded, 43°C
0	2.8 ± 0.29[a]	2.8 ± 0.29[a]	2.8 ± 0.29[a]	2.8 ± 0.29[a]
4	2.5 ± 0.45[a]	2.8 ± 0.6[a]	1.75 ± 0.82[a]	2.5 ± 0.53[a]
48	1.5 ± 0[b]	1.67 ± 0.42[b]	1.63 ± 0.48[b]	1.38 ± 0.52[b]

Table 4. The differences in means of intensity of immunostaining for type I procollagen (± standard deviation) among different groups were measured by analysis of variance. The range of intensity was from 0 (no staining) to 3 (maximum staining). Dissimilar letters indicate significantly different groups at *P<0.05*.
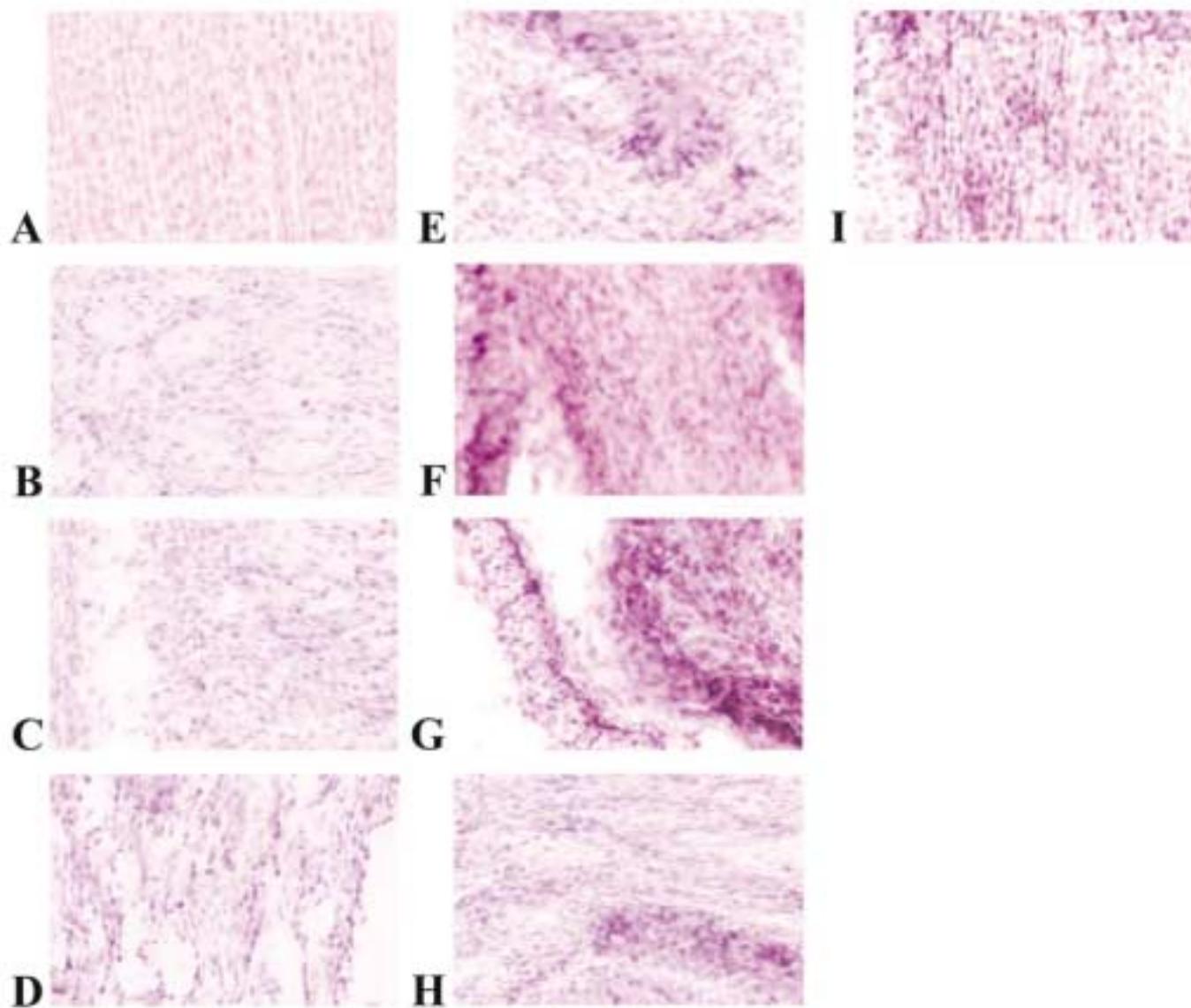
Figure 3. Expression of CTGF mRNA. Almost no CTGF mRNA was visualized by ISH in tendons at the time of removal (A). A moderate increase in expression was observed in intact (B) and wounded (C) tendons maintained at 37°C and in intact (D) and wounded (E) tendons kept at 43°C tendons for 72 hours. CTGF mRNA was strongly expressed in intact (F) and wounded (G) tendons maintained at 37°C and in intact (H) and wounded (I) tendons at 43°C after 144 hours in culture. Counterstained with hematoxylin. Magnification x 200.

for 1 hour (Table 2). The level of Hsp47 mRNA expression at 12 hours (Table 2) was significantly decreased when compared with expression at 1 hour, but not when compared with expression at removal time. With longer culture time it continued diminishing. At 48 hours only mild expression, significantly decreased when compared with expression at 1 hour, was observed in all tendons (Table 2). Hsp47 mRNA expression became undetectable in all tendons at 96 hours (data not shown). No staining was observed when the antisense probe was replaced with the sense probe (data not shown).

Expression of CTGF mRNA in tendon explants

CTGF mRNA was undetectable during the first 48 hours of *in vitro* incubation (Figure 3A, Table 3), then its level gradually increased. At 72 hours a significant moderate increase in expression was noted in all tendons, there was no effect of additional injury or temperature (Figures 3B and C, Table 3). Cultures maintained at 43°C for 72 hours revealed significantly higher levels of expression of CTGF mRNA (Figures 3D and E, Table 3). Another significant increase in the level of

CTGF mRNA occurred at 96 hours (Figure 3F-I, Table 3) and continued until the experiment was terminated after 144 hours of culture at both temperatures. It was located exclusively in the cytoplasm. The expression was strongest in synovial cells of the sheath. The cartilage present in some tendons near the insertion site showed only little staining (data not shown).

Collagen in tendon explants

Gastrocnemius tendons from 18-day old chicken embryos consist of elongated tendon fibroblasts organized along the vertical axis formed by parallel fibers of collagen. The embryonal tendon is fairly cellular and the type I procollagen fibers are short (Figure 4). Type I procollagen was identified using immunohistochemistry as short thick fibers that were dispersed throughout the tendon. No staining was noted when irrelevant primary antibodies were used (data not shown). The intensity of immunostaining for type I procollagen was strong immediately upon excision and remained so at 4 hours of culture at both temperatures (Figures 4A and B, Table 4). It decreased significantly at 48 hours of culture in all tendons (Figure 4C, Table 4) and remained low until at the end of the experiment 1 week after excision (data not shown). No changes in collagen fiber morphology were noted. We did not observe any discernible changes in type III collagen expression (data not shown). No healing processes, including cell proliferation or fiber production, were observed at any time during the experiment either at sites of excision or when additional cuts were made.

Discussion

We have shown that the level of immunostained type I procollagen and expression of TGFβ and Hsp47 mRNAs in chicken embryonal tendon explants decreased with the length of *in vitro* culture and that the impact of elevated (43°C) culture temperature and the presence of additional injuries was minimal. The insignificant changes in Hsp47 mRNA level in tendons maintained for 1 hour at 43°C appears to be paradoxical: after all as a classical heat shock protein Hsp47 expression increases during the first several hours of temperature increase[11,20,29]. In our experiments the decrease in Hsp47 mRNA was noticeable only after 12 hours of culture, and it was independent of environmental temperature. It is likely that tendon excision or removal acted as a potent stimulus of Hsp47 expression at the time of removal and persisted during the first 12 hours of culture. Our results differ from those by Newman Keagle et al. who showed no presence of Hsp47 in intact rodent skin and a gradual increase in its levels by immunohistochemistry and Western blotting in the dermis over the period of 2 weeks after linear skin wounding[30]. Whether those differences can be attributed to species and tissue differences, or differences in experimental conditions (*in vitro* vs. *in vivo* environment) is not clear. One, perhaps important, difference was that we measured only the mRNA expression for TGFβ, Hsp47 and

CTGF, whereas Newman Keagle et al. used immunohistochemistry and Western blotting for Hsp47 detection[30].

TGFβ expression underwent changes similar to changes in Hsp47 expression. The decrease in expression of TGFβ over time was only slightly higher with wounding or increased temperature. Conditions that affect Hsp47 and TGFβ levels may also affect collagen content or structure[3,31]. Our data show a decrease in the content of immunostained type I procollagen only after 48 hours of explant culture. This is most likely due to the fact that changes in mRNA levels occur much more rapidly than changes in the level of protein synthesis. We found that immunostaining for type I procollagen on frozen sections was more intense than detection of type I procollagen by Western blotting (data not shown). We attribute this relative lack of staining by Western blotting to masking of epitopes on the procollagen molecule during processing[22].

The significant increases in CTGF mRNA expression were due to the length of explant culture but not due to temperature increases or wounding. The late onset (after 72 hours of culture) of CTGF expression noted in these experiments is consistent with observations in other models of repair. Although TGFβ is the only known inducer of CTGF, with the possible exception of glucocorticoids[32], an interval of 2-14 days between TGFβ peak expression and CTGF expression has been noted in other tissues[8,33]. In our experiments CTGF expression remained at high levels after 7 days of *in vitro* incubation. We presume that the induction of TGFβ expression by tendon removal led to delayed CTGF expression. To determine whether differences in CTGF expression would have been apparent in subsequent days would have required additional later time points. In murine models of cutaneous repair, there does not appear to be a direct quantitative correlation between amount of TGFβ and CTGF expression. Rather, a local threshold level of active TGFβ seems to be required for CTGF induction[7]. Recruitment of additional fibroblasts by CTGF and their proliferation further enhance its expression and correspond to the lag between TGFβ stimulation and CTGF peak expression.

Human TGFβ1 probes and TGFβ1 antibodies are known to cross-react with several chicken TGFβ mRNA and protein isoforms, respectively[24,34]. Our data indicate that we detected mostly chicken TGFβ2 and TGFβ4 mRNAs in our ISH experiments. Northern blotting revealed two major bands, one migrating at 4.1 kb is most likely TGFβ2 mRNA, whereas the one migrating at 1.8 kb is likely TGFβ4 mRNA. As observed by Jakowlew et al., TGFβ2 and TGFβ4 are the main TGFβ isoforms expressed in chicken embryo fibroblasts and chondrocytes[25]. Our recent results indicate that TGFβ4 plays the role of, and may even be TGFβ1 in chicken tendon cells and it induces the production of type I procollagen and Hsp47 in these cells[26]. Therefore, it is likely that our TGFβ mRNA probe detected mostly TGFβ4 or 1 in examined tendon explants.

TGFβ is a potent inducer of collagen synthesis. This effect is likely direct[2,3] and indirect through CTGF[6]. It has also been shown that TGFβ stimulates DNA synthesis in the ten-
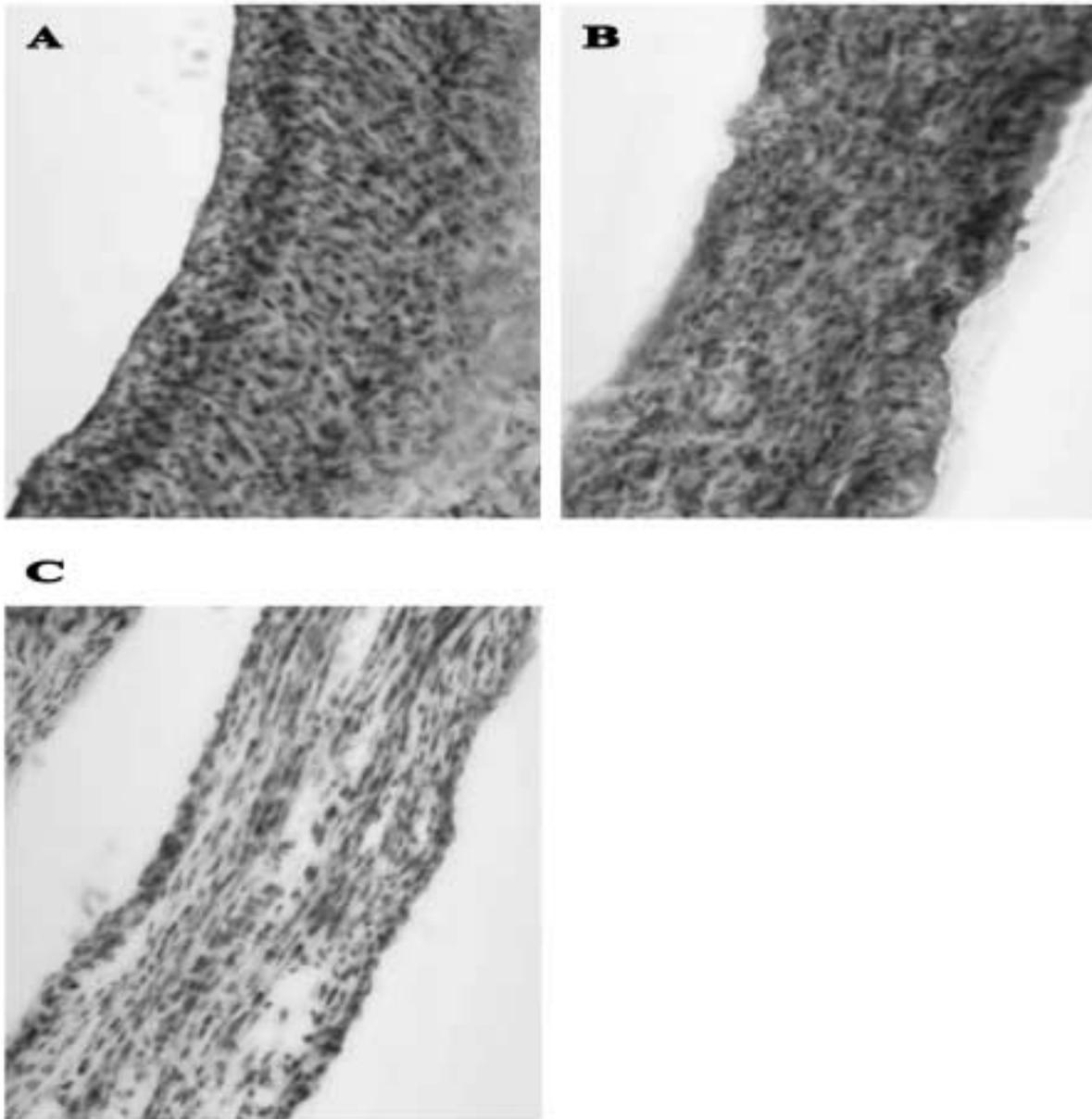
Figure 4. Immunohistochemistry for type I procollagen showed relatively short, moderately stained fibers (arrows) of procollagen I after 4 hours of culture in intact tendons kept in culture at 37°C (A) and at 43°C (B). The staining was mild after 72 hours of culture at 43°C (C). Magnification x 400.

don[35]. Besides TGFβ and CTGF, insulin-like growth factor type I (IGF-I) has been identified in the tendon[36,37] and been shown to stimulate proliferation of tendon fibroblasts[36] IGF-I most likely plays an important role in tendon healing though it does not appear to have an effect on type I and type III collagen synthesis[37].

It is possible that the lack of mechanical loading rather than the duration of *in vitro* culture was responsible for the decreased presence of procollagen and expression of collagen regulating genes. Banes et al. have documented that mechanical load stimulates expression of numerous genes in avian flexor tendon cells[38]. Loading has been shown to increase not only mRNA expression of type III collagen[39] but also of matrix metalloproteinases in rabbit Achilles tendon[39-41]. The increase in matrix metalloproteinases indicates either remodeling or injury to the tendon. The potentiation of stretch-dependent induction of matrix metalloproteinases by interleukin-1β indicates that injury secondary to loading (e.g., exercise) may turn a remodeling process into degradation and degenerative changes in tendon[41]. Butt and Bishop have shown that mechanical stress potentiated TGFβ-induced collagen synthesis in cardiac fibroblasts[42].

We realize that even the so-called uninjured or control tendon explant underwent an excision which in itself does constitute an injury. It is, therefore, likely that additional cuts did not lead to changes in growth factor, Hsp and collagen levels. The apparent lack of proliferative or inflammatory reaction in wounded tendon can also be attributed to *in vitro* situation, though the results are also consistent with lack of a significant inflammatory response during the first post-injury week in tendon repair *in vivo*[43,44].

In summary, we have shown that *in vitro* explant culture lead to downregulation of collagen-modulating genes, such as TGFβ and Hsp47, lower content of immunostained type I procollagen and delayed upregulation of CTGF. Interestingly, elevated culture temperature (43ºC) and additional wounding did not appear to have major effects. It is likely those effects would be noticeable only in tendons undergoing loading. The presence of TGFβ, IGF-I and other growth factors in FBS supplementing culture medium might have also contributed to the relatively minor changes in levels of collagen and in expression of Hsp47 and TGFβ.

Future experiments are needed that would establish whether weight-bearing tendons would undergo similar changes in levels of collagen-regulating gene expression and in synthesis of collagen during periods of *in vivo* inactivity, and whether these changes would also impart tendon ultrastructure and biomechanical function. We conclude that *in vitro* culture over the period of several days decreases the expression of TGFβ and Hsp47 in avian tendon explants through adaptation to new conditions, and may thus differ from short-term effects of increased temperature (acting as a "heat shock") on Hsp47 expression.

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