Molecular Effects of Sodium Hyaluronate on the Healing of Avian Supracoracoid Tendon Tear: According to In Situ Hybridization and Real-Time Polymerase Chain Reaction

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ABSTRACT: Hyluronic acid (HA) on tissue healing has been controversial. We examined the molecular pharmacology of HA injection at the suture site in an acute model of supracoracoid tendon laceration using chickens, an injury of a nonweight-bearing joint considered similar to the human shoulder. Expression of mRNAs encoding α I (I) and αI (III) procollagens was localized using in situ hybridization (ISH). Intensities of mRNA expression for α I (I) and α I (III) procollagens, transforming growth factor-β1 (TGF- β1), basic fibroblast growth factor (bFGF), and insulin-like growth factor (IGF) were determined by quantitative reverse transcription-polymerase chain reaction (RT-PCR). Histologically, chickens with HA injection (HA group) showed early restoration of continuity at the laceration site than saline-injection controls (saline-injection group). By ISH, the expression rate of cells at the lesion site that contained α I (I) and α I (III) procollagen mRNAs were somewhat higher in the HA group than in the saline-injection group. By RT-PCR, the HA- and saline-injection groups showed no significant difference in expression of α I (I) and α I (III) procollagen mRNA between weeks 1 and 6. The saline -injection group exhibited significant decrease in TGF-β1 expression between weeks 1 and 3, and in bFGF expression between weeks 1 and 2; however, the HA group showed no such decrease. As for IGF, no difference was appreciable in both groups between weeks 1 and 6. A single injection of HA could cause earlier restoration of continuity at the lacerated site of the supracoracoid tendon. © 2006 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 25:173–184, 2007

Keywords: sodium hyaluronate; rotator cuff tear; real-time reverse transcription-polymerase chain reaction (RT-PCR); tendon healing; procollagen

INTRODUCTION

Hyaluronic acid (HA) is abundant in cerebrospinal fluid and in the extracellular matrix of soft connective tissues, particularly in connective tissue undergoing development, regeneration, or reconstruction at times of marked cell proliferation and migration.[1] Accordingly, the importance of HA during early stages of repair processes at joints has been investigated several times,[1–6] but typically in connection with flexor tendons, the Achilles tendon, the knee, or ligaments.[2–4,6] We know of no report directly concerning HA in an experimental model of healing in rotator cuff tears.

Clinical use of HA in management of shoulder joint disease commonly entails intraarticular or intrasynovial injection; beneficial effects have been established in frozen shoulder as well as rotator cuff injury.[7] Postulated mechanisms of benefit mostly have focused on the viscosity of HA causing improvement of sliding properties or preventing adhesions.[2–4,6] Wiig and Abrahamsson[8] examined the effect of HA on cell proliferation and matrix production using rabbit deep flexor tendons, but

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the molecular pharmacology of HA in damaged rotator cuff tissues has not yet been investigated. We have studied the repair process in the rotator cuff of a nonweight-bearing joint by examining an avian acute model. Using in situ hybridization (ISH), Kobayashi et al.[9] demonstrated that the tendon reparative process progressed from the bursal to the articular side in this model. This sequence of repair is considered to be similar to that in human rotator cuff rupture.[10–12]

In the current study, we followed the healing process in this rotator cuff acute tear model over time after administration of HA. Simultaneously, we localized expression of α I (I) and α I (III) procollagen mRNAs using in situ hybridization (ISH), and quantitated mRNAs encoding α I (I) and α I (III) procollagens, transforming growth factor-β1 (TGF-β1), basic fibroblast growth factor (bFGF), and insulin-like growth factor (IGF),[13–16] by quantitative PCR. All of these gene products have been implicated in neogenesis of tendon and soft tissues. The primary objective of the present study was to better understand the pharmacologic action of HA in a damaged tendon at the shoulder joint, particularly the molecular underpinnings of its clinical benefit.

MATERIALS AND METHODS

Supracoracoid Tendon Tears in Chickens

Mature white leghorn chickens ($n = 40$; age, 28 months; weight, 2.8–3.0 kg) were anesthetized with pentobarbital (30 mg/kg i.m.). An incision was made on the dorsal aspect of the right shoulder. A space between the major and minor deltoideus muscles was enlarged; then, the thin bursa-like structure overlying the supracoracoid tendon was partially incised, from approximately 10 to 18 mm proximal to the insertion. A transverse full-thickness laceration 3 mm in length was created in the central portion of the tendon, which was 5 mm in width, using a chisel 3 mm wide. The extent of the laceration was approximately 60% of tendon width. The laceration was located 15 mm proximal to the insertion, approximately 10 mm distal to the musculotendonous junction. Selection of the laceration site took account of anatomic differences from the human shoulder. We confirmed that the articular cartilage of the humeral head was exposed through the tendon laceration site. The gap between proximal and distal sides of the laceration was closed with one 4-0 nylon simple suture, following the procedures described by Kobayashi et al.[9] (Fig. 1). The bursa-like structure was closed in an atraumatic manner with two simple 4-0 nylon sutures to prevent leakage before injecting 0.5 mL of 1% HA hyaluronan into the bursa-like structure(ARTZ, pH 7.2; molecular

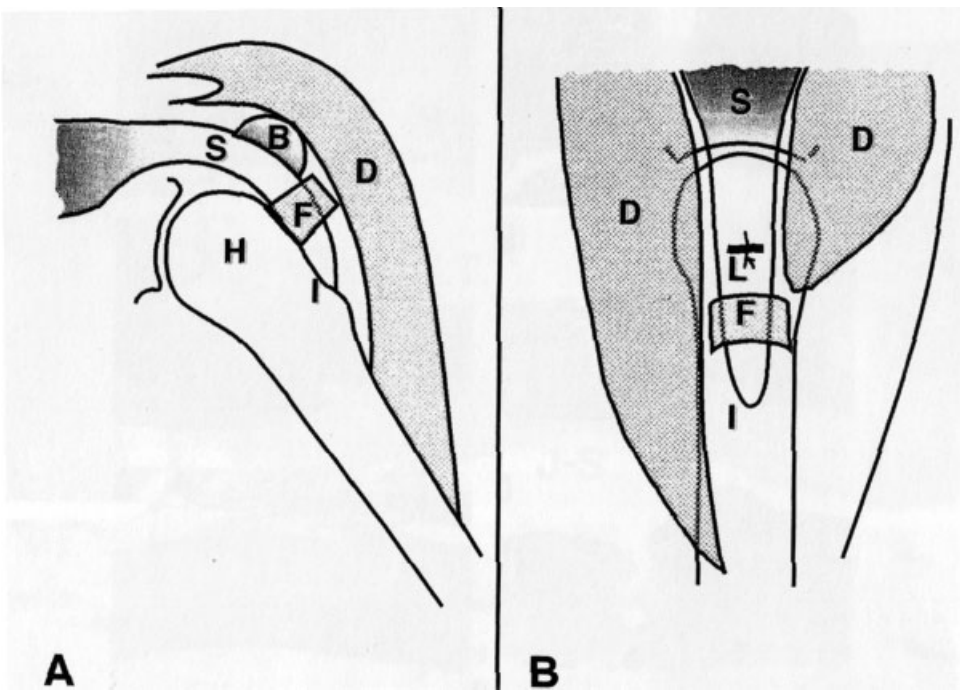

Figure 1. (A) A schematic drawing of anatomy of an avian supracoracoid tendon and its environs (longitudinal section). As human supraspinatus tendon, the avian supracoracoid tendon faces the bursa-like structure superiorly, and the shoulder joint capsule inferiorly. (B) Lacerated and sutured tendon. B, Bursa-like structure; D, deltoid muscle; F, fibrous pulley; H, humeral head; I, insertion of the tendon; L, lacerated and suture site; S, supracoracoid tendon (cited from ref. 9).

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weight, 9×10^5; Kaken Pharmaceutical, Tokyo, Japan). This group was called the HA group.

In the left shoulder, all procedures were performed along the same manner of HA group except HA injection; 0.5 mL of phosphate-buffered saline (PBS; pH 7.8) was injected into the repaired bursa-like structure instead of HA. This group was called the saline-injection group.

All procedures were performed under sterile conditions. No immobilization around the shoulder girdle was used, and chickens were allowed to move freely in their cages after surgery. Ten chickens each were killed after 1, 2, 3, and 6 weeks; supracoracoid tendons were harvested together with surrounding tissues. The Institutional Review Board approval was obtained prior to performing this study.

Tissue Preparation for Microscopic Examination Including In Situ Hybridization

The 40 supracoracoid tendons and surrounding tissue from 20 chickens used for microscopic assessment including ISH were transferred immediately to sterile tubes containing 10% neutral buffered formalin (Wako Pure Chemical, Osaka, Japan) and fixed for 48 h at 4°C. The fixed tissues were dehydrated and embedded in paraffin (Wako Pure Chemical). Specimens were sectioned longitudinally at a thickness of 6 μm and affixed to silane-coated slides (Matsunami Glass, Osaka, Japan). The slides were dried at 42°C for 24 h.

Histologic Analysis

Adjacent sections including the central portion of the tendon laceration site were stained with hematoxylin and eosin (H&E) or Azan blue for examination using an Olympus BH-2 microscope (Olympus Optical, Tokyo, Japan).

In Situ Hybridization

Oligonucleotide probes used for the detection of α I (I) and α I (III) procollagen mRNAs were as described by Kobayashi et al.[9] Antisense probes and corresponding sense probes were synthesized and 3′-end-labeled with digoxigenin-11-deoxyuridine triphosphate (Boehringer Mannheim, Germany) using terminal deoxynucleotidyl transferase (Boehringer Mannheim). They were used without further purification.

In situ hybridization was performed according to methods originally established by Hamada et al.[10,17,18] and Kobayashi et al.[9] Briefly, sections were rehydrated and pretreated by sequential immersion in 0.2 N hydrochloric acid (HCl) for 10 min at room temperature and then proteinase K (10 μg/mL, Sigma Chemical, St. Louis, MO) in 0.1 M hydroxymethyl aminomethane (Tris)-HCl (pH 7.5) for 20 min at 37°C.

The hybridization mixture (50 μL) containing the labeled oligonucleotide probe (300 pg/μL) was spread over the pretreated dried sections on the glass slides. Sections were incubated at 21°C overnight within a humid chamber containing 50% formamide.

To detect hubridized probes, section then were incubated sequentially with the following reagents for 30 min each at room temperature: mouse monoclonal antidigoxin (1:10 000; Sigma Chemical); biotinylated antimouse F (ab′) 2 fragment (1:200; Dako, Mississauga, ON, Canada); and avidin conjugated with alkaline phosphatase (1:100; Dako).

Probe-mRNA hybrids bound to alkaline phosphatase were visualized with 340 μg/mL of 4-nitroblue tetrazolium chloride (Boehringer Mannheim) and with 170 μg/mL of 5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine salt (Boehringer Mannheim) as a substrate at room temperature in darkness. Substrate reaction times were standardized at 60 min to permit visual comparisons of the staining reaction. Sections were covered with Crystal/Mount (Biomeda, Foster City, CA) and mounted with Eukitt (Kindler, Freiburg, Germany).

Sections were studied under an Olympus BH-2 microscope. Cells with positive signal (PSC) were counted in nine zones including the superficial (bursal aspect), middle, and deep (articular aspect) tendon layers proximal to the laceration; in intervening (lesioned) tissue; and distal to the laceration. Cells exhibiting hybridization labeling were counted in five 2500-μm^2 areas (50×50 μm) randomly selected from within each of the nine zones.

Control Studies

The following four control experiments were performed on adjacent sections to evaluate probe specificity and to validate the in situ hybridization protocols for use with these tissues.

Probe Omission

Sections were incubated with hybridization buffer only and then subjected to all subsequent steps.

Competition Test

Sections were incubated with labeled probe in the presence of a 100-fold excess concentration of unlabeled probe.

RNase Treatment

Prior to incubation with a labeled probe, sections were pretreated with 100 μg/mL of RNase A (Boehringer Mannheim) in Tris-HCl (pH 7.5) for 1 h at room temperature.

Incubation with the Sense Probe

Sections were incubated with the labeled corresponding sense oligonucleotide probe.

RNA Isolation and RT

The 40 supracoracoid tendons and surrounding tissues harvested at varying time points from the remaining 20 chickens that underwent the tendon laceration

procedure were stored frozen at −80°C. After thawing at room temperature, the tissue samples were homogenized with a Polytron homogenizer in a 4.0-M guanidinium thiocyanate buffer. The tissue homogenate then was centrifuged through a cushion of 5.7 M CsCl at 32,000 rpm at 20°C for 16 h. After phenol-chloroform treatment, total RNA was extracted by ethanol precipitation. The total RNA was stored at −80°C. RT was performed at 24°C for 10 min; at 42°C for 60 min, and at 100°C for 5 min using 1 μg of total cellular RNA, 100 pM random primer, (Boehringer Mannheim), and reverse transcriptase (GIBCO BRL, Rockville, MD).

Quantitative PCR

For real-time PCR, the ABI Prism 7700 Sequence Detection System was used with TaqMan Core Reagent (Applied Biosystems, Foster City, CA). We designed probes and primers sequences according to the referenced complete mRNA codes of chicken by previous reports.[19–23] PCR conditions included initial denaturation for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. A PCR assay for 18S ribosome labeled with a different fluorochrome was performed in the same samples as an internal control. As a positive control to construct calibration curves, mRNAs of interest were amplified from mRNA extracted from the ovarian cells of white leghorn chickens. Then, the expression level of (Cell containing C_T value − y)/m, formulated by incorporating the impacts of the gradient (m) of the calibration curve derived from the C_T value and also the y-intercept value (y), was calculated for comparisons of expression frequencies between groups.

Statistical Analysis

Fisher's protected least significant difference (PLSD) test was applied for comparisons of expression frequencies between groups. p-Values less than 0.05 were considered to indicate statistical significance.

RESULTS

Histologic Study

The supracoracoid tendon has a bursal aspect, as well as an articular aspect covering a thin joint capsule and facing the humeral head. Because a distinction cannot be made in histologic sections between the bursal floor and the surface of the tendon, or between the joint capsule and the articular surface of the tendon, these interfacing areas are designated as peritendon areas.

Saline-Injection Group

At week 2, cells proliferating in the epitenon on the bursal aspect were seen migrating to the articular surface as if to fill the gap in the tendon. Specimens in which continuity was restored in all layers were four of five per time point starting at week 6. Tissues that filled the gap included significant numbers of fibroblasts, new blood vessels, and reticular fibers (Fig. 2).

HA Group

At week 2, cells proliferating in the epitenon on the bursal aspect were seen migrating to the articular aspect as if to fill the tendon gap. Specimens in which continuity was restored in all layers were one at week 2, two at week 3, and four at week 6. Tissues that filled the gap included significant numbers of fibroblasts, new blood vessels, and reticular fibers. Fibroblasts in the superficial layer of the intervening tissue were arrayed more densely than in the saline-injection group, and surrounding collagen fibers exhibited greater maturity.

In Situ Hybridization

In the negative control experiments for α I (I) and α I (III) procollagen probes (probe omission, probe competition, RNase treatment, and incubation with the sense probe), positive signal cells (PSC) were not detected (data not shown).

Saline-Injection Group

Expression of α I (I) Procollagen mRNA

PSC were detected among fibroblasts and tendon cells beginning at week 2 (34.2 cells/2500 μm^2); no PSC were detected at week 1. Numbers of PSC increased between weeks 3 (171.6 cells/2500 μm^2) and 6 (244.1 cells/2500 μm^2). Regarding the total number of PSC at a given time point as 100%, the greatest number of PSC at week 2 was found in the superficial layer of the intervening tissue (55%), followed by the middle layer (23%) and the deep layer of the intervening tissue (12%). Between the period of weeks 3 and 6, the amount of expression increased in the deep layer of the intervening tissue (26%), while at week 6, expression was uniform in all layers of intervening tissue (77%) (Fig. 3A).

Expression of α I (III) Procollagen mRNA

PSC were detected among fibroblasts and tendon cells beginning at week 2. No PSC were detected at week 1. Numbers of PSC increased between weeks 3 (109.5 cells/2500 μm^2) and 6 (138 cells/2500 μm^2). At week 2 the most common site for PSC was the superficial layer of the intervening tissue (38%), followed by the superficial layer of the tendon

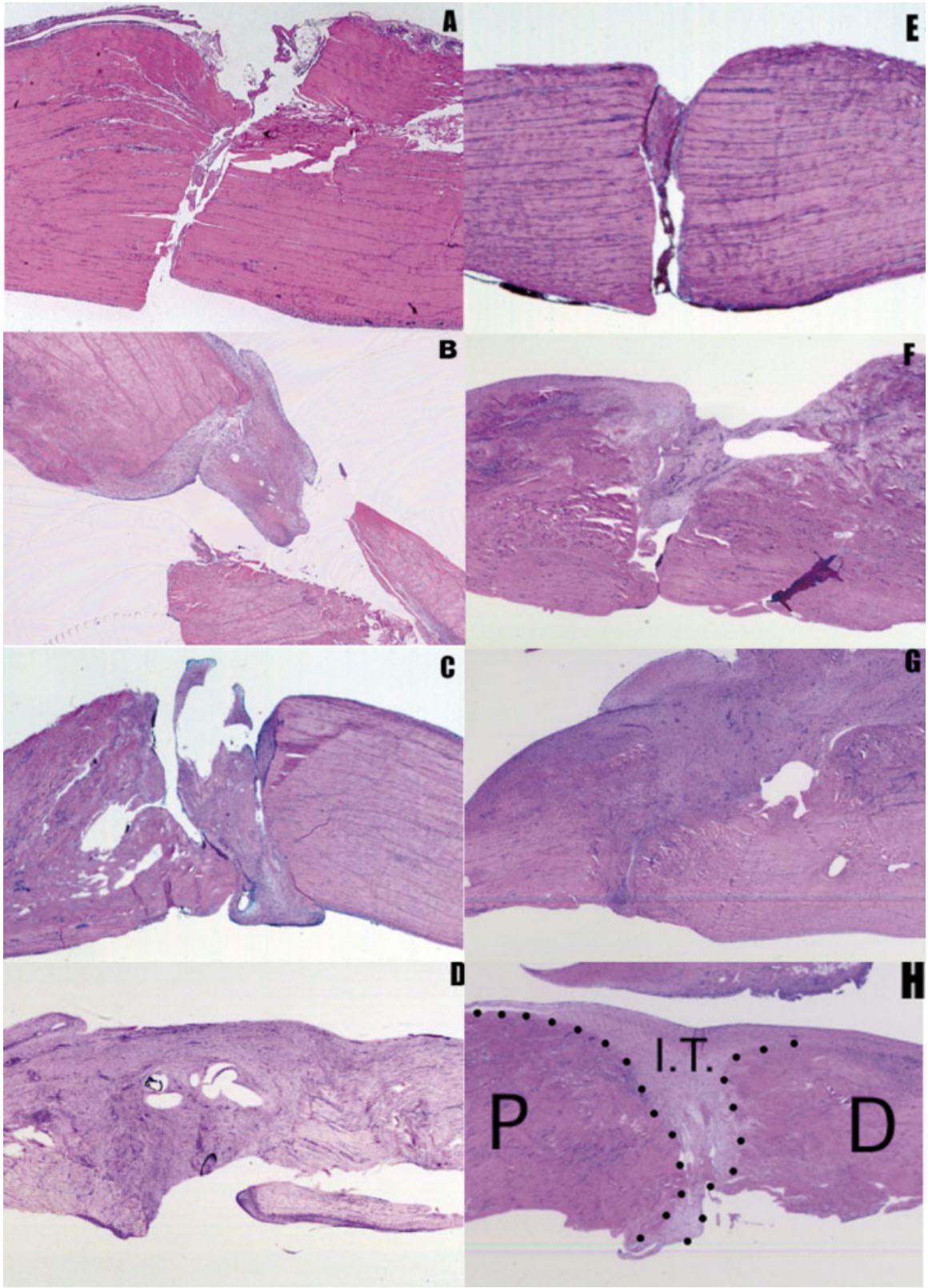

Figure 2. (A) Saline-injection group week 1; (B) saline-injection group week 2; (C) saline-injection group week 3; (D) saline-injection group week 6; (E) HA group week 1; (F) HA group week 2; (G) HA group week 3; (H) HA group week 6. The intervening tissue was denser compared with the saline-injection group, and the surrounding collagen tissue also exhibited a higher level of maturity. P, proximal tendon stump (original); I.T., intervening tissue; D, distal tendon stump (original). (Original magnification ×5).

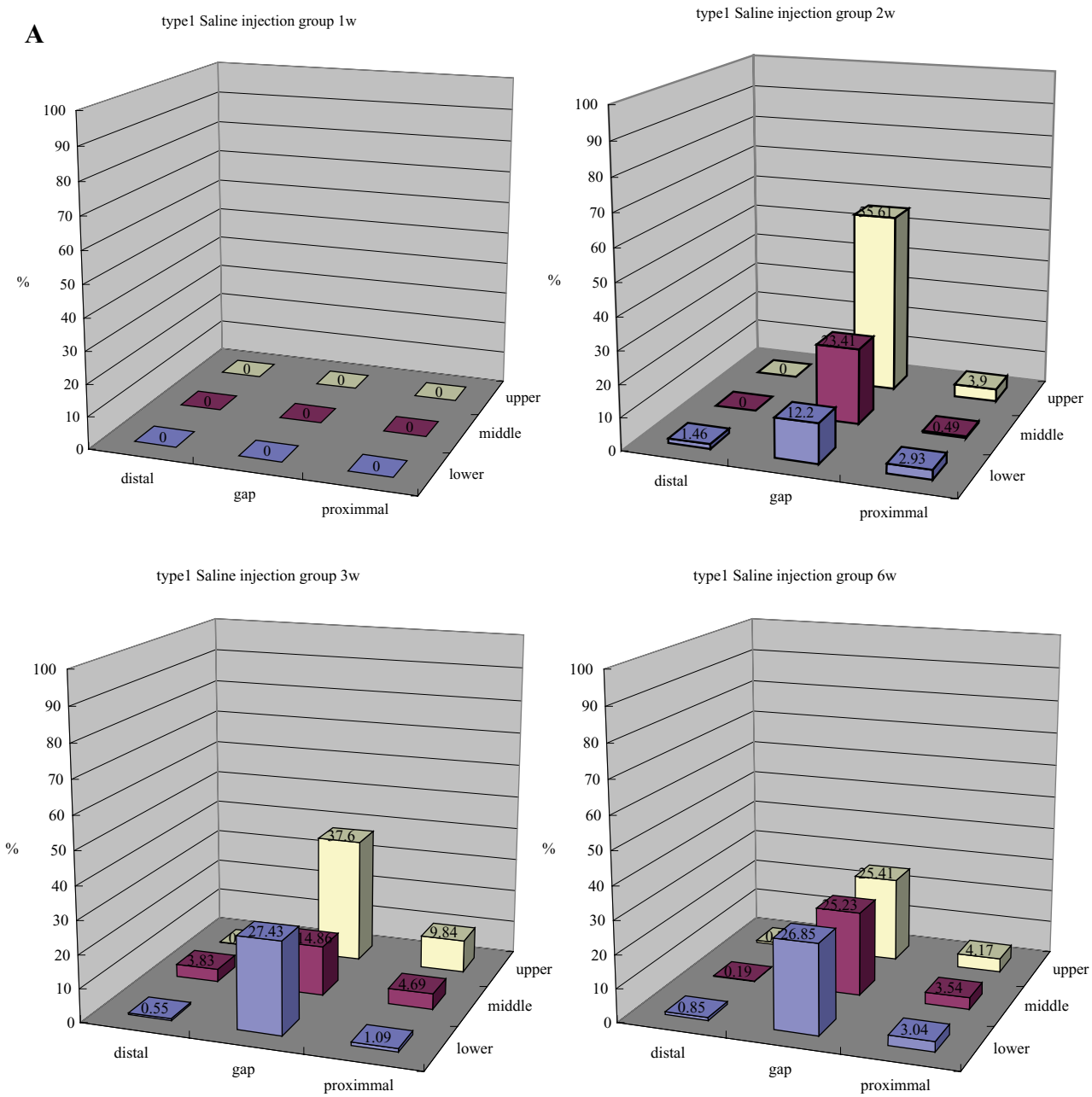

Figure 3. Localization ratio of PSC in nine zones. The graph showing the average percentage of the number of PSC for α I (I) procollagen mRNA and α I (III) procollagen mRNA. (A) α I (I) procollagen mRNA of the saline-injection group; (B) α I (III) procollagen mRNA of the saline-injection group; (C) α I (I) procollagen mRNA of the HA group; (D) α I (III) procollagen mRNA of the HA group.

proximal to the lesion (22%) and then the middle layer of the intervening tissue (19%). Between weeks 3 and 6, PSC were essentially confined to the intervening tissue, with increased numbers in the deep layer. At week 6, expression was more similar between layers of the intervening tissue (superficial, 31%; middle, 37%; and deep, 25%. Compared with the distribution for expression of α I (I), more expression of α I (III) was detected in the superficial layer of the tendon proximal to the lesion (Fig. 3B).

HA Group

Expression of α I (I) Procollagen mRNA

PSC were detected among fibroblasts and tendon cells. PSC began to emerge at week 2 (123.9 cells/2500 μm^2), having been undetectable at week 1.

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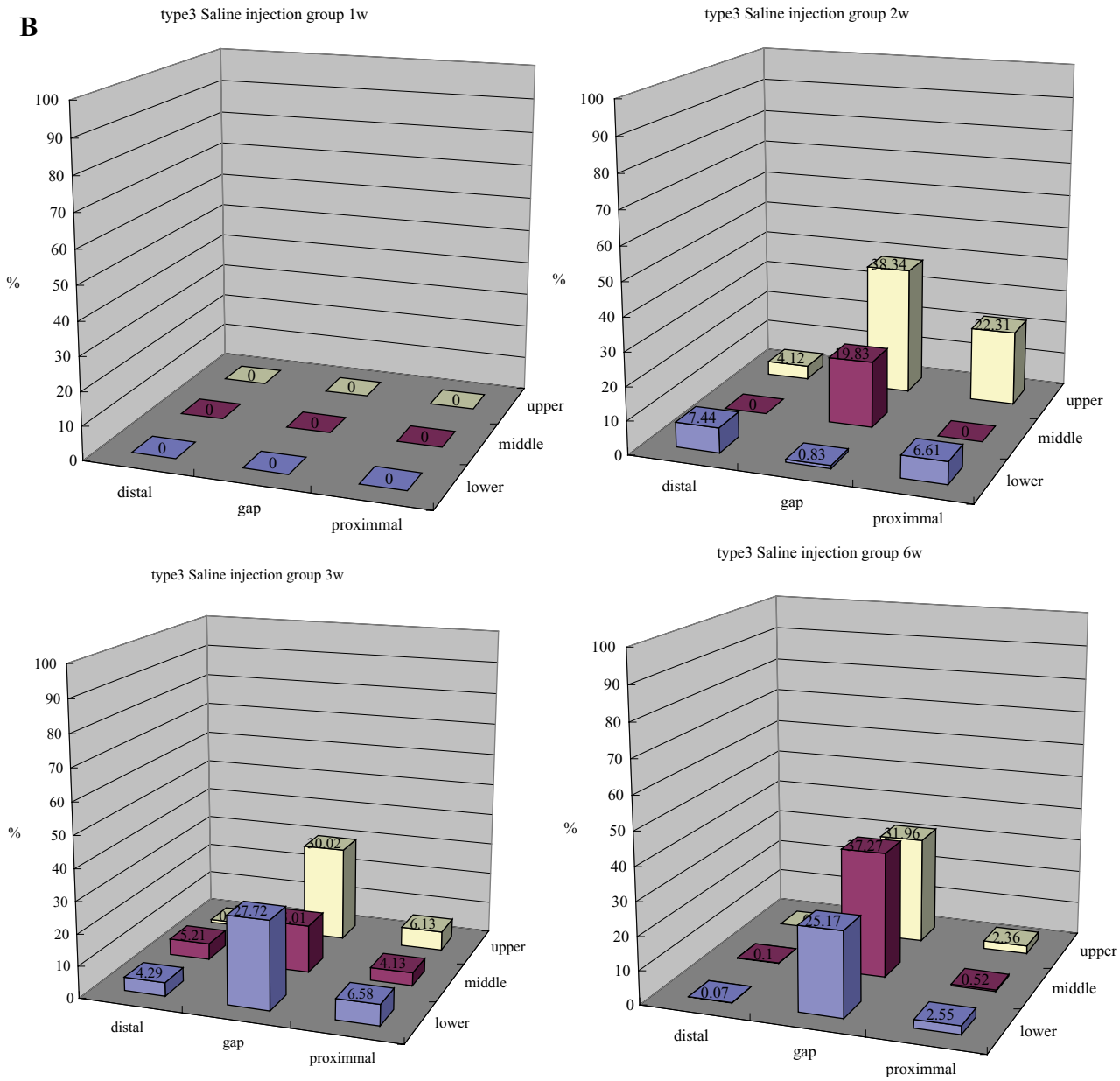

Figure 3. (*Continued*)

PSC suddenly increased at week 3 (320.5 cells/2500 μm^2), when they attained greatest numbers. Between weeks 3 and 6, numbers of PSC decreased rapidly. At week 2, most PSC were localized in the superficial layer of the intervening tissue (46%). Further, compared with the PBS group, more intense localization of PSC was observed in the superficial layer of the tendon distal to the lesion (20%). At week 3, the PSC were found most commonly in the superficial layer of the intervening tissue (34%). By week 6, the number of PSC was very small (31.2 cells/2500 μm^2), mostly localized to the superficial layer of the intervening tissue (30%) and the middle layer of the intervening tissue (31%). Compared with the saline-injection group, the HA group showed greater increase in expression of PSC in superficial and middle layers of the intervening tissue (Fig. 3C).

Expression of α I (III) Procollagen mRNA

PSC were detected among fibroblasts and tendon cells. Moderate numbers of α I (III) PSC were detected among fibroblasts and tendon cells proximal and distal to the lesion. PSC first were detected at week 2. The greatest number of PSC was detected

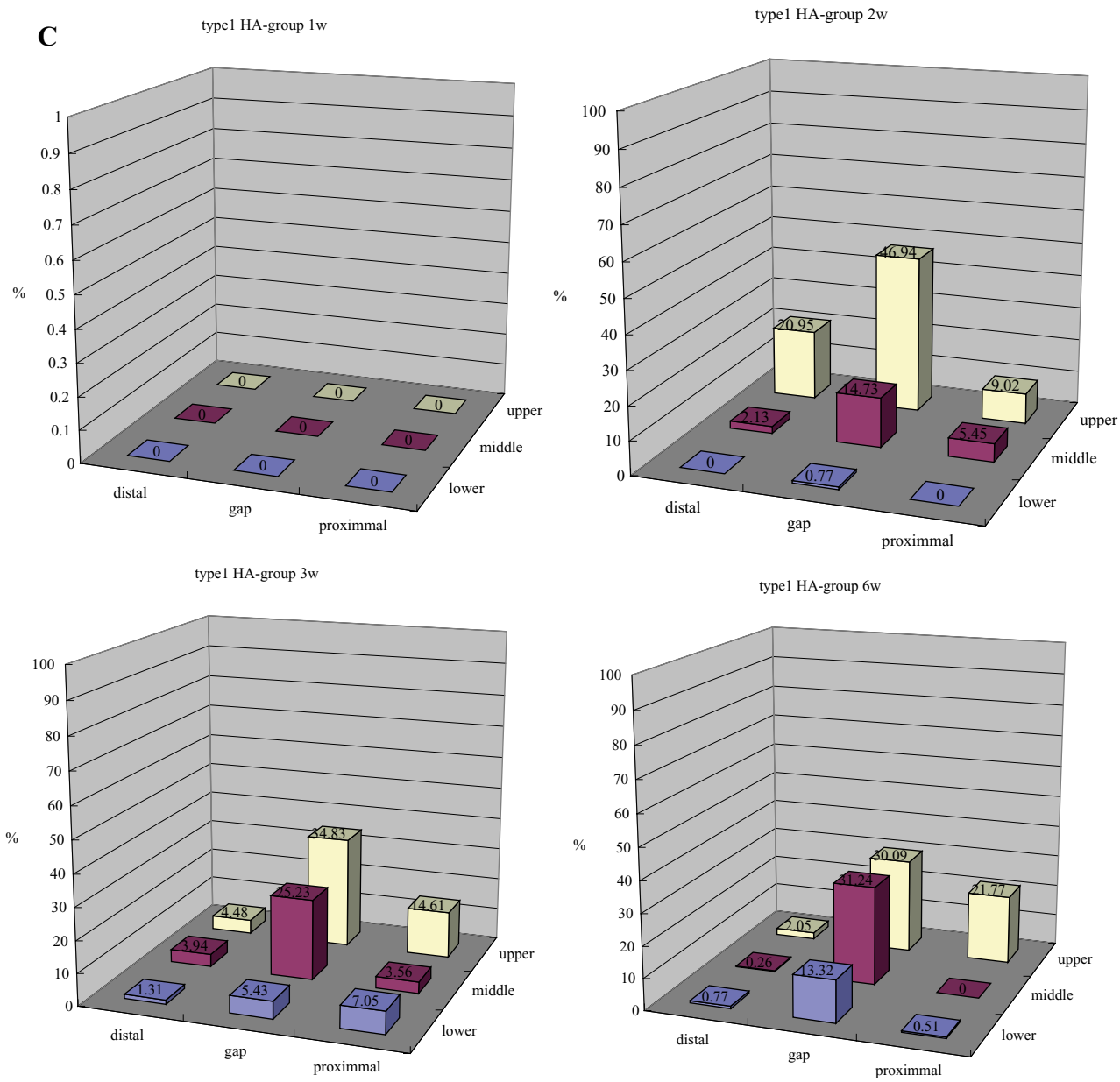

Figure 3. (*Continued*)

at week 3 (220.8 cells/2500 μm^2), although no PSC were detected at week 6. At week 2, most PSC were detected in the superficial layer of the intervening tissue (37%), followed by the middle layer of the intervening tissue (22%) and then by the superficial layer of the distal part of the tendon (17%). At week 3, PSC were found most abundantly in the superficial layer of the intervening tissue (42%), followed by the middle layer of the intervening tissue (25%). Compared with the saline-injection group regarding the expression distribution of PSC, greater expression rate of PSC was found in the superficial and middle layers of the intervening tissue, as had been true for α I (I) procollagen mRNA (Fig. 3D).

RT-PCR Studies

Between weeks 1 and 6, no significant difference was observed in expression of α I (I) procollagen mRNA between the HA- and saline-injection groups. Similarly, between weeks 1 and 6, no significant difference was noted in the expression of α I (III) procollagen between HA and saline groups. In the saline-injection group, TGF-β1 exhibited a significant decrease at week 3 compared with week

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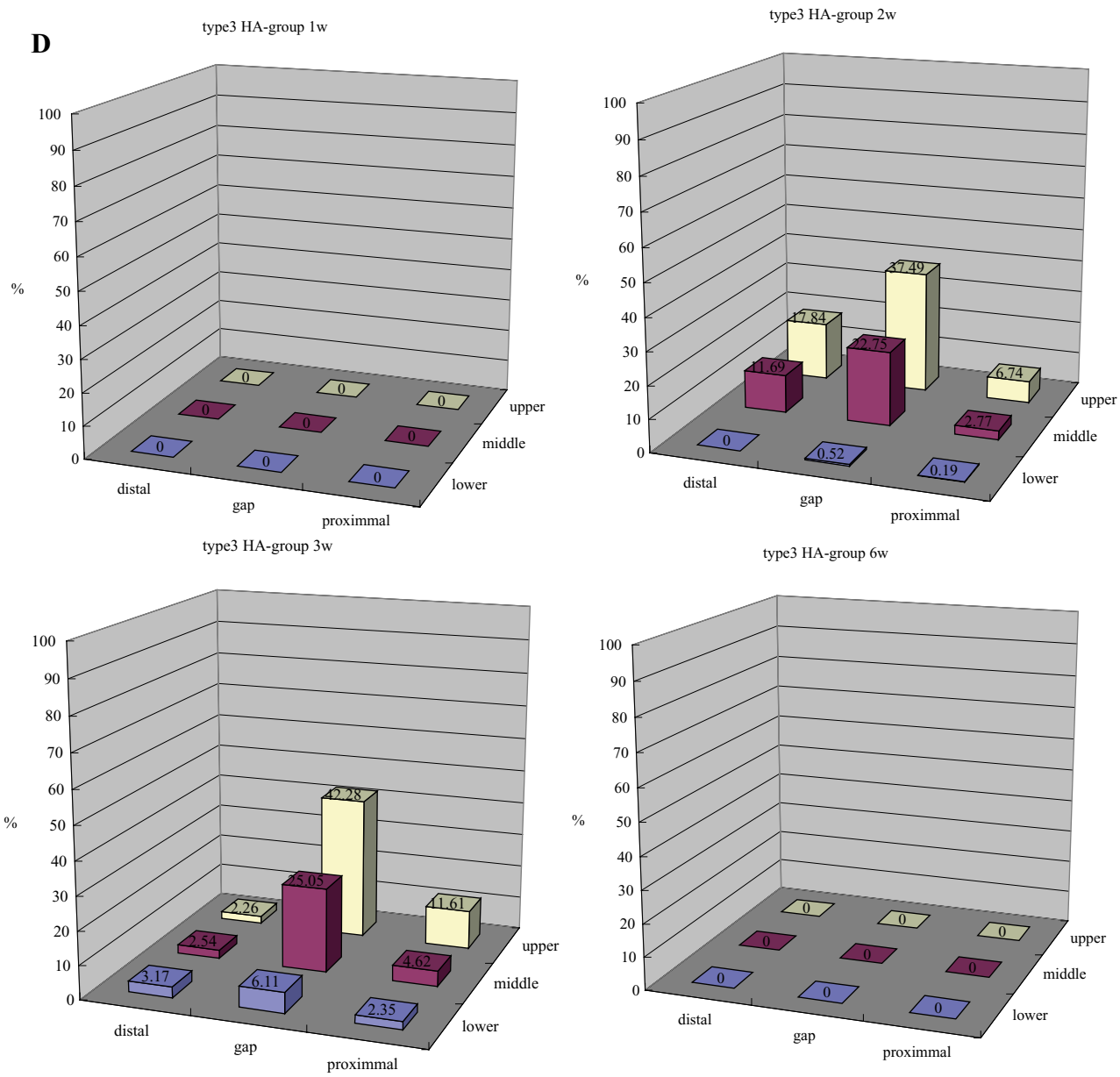

Figure 3. (*Continued*)

1, while in the HA group no significant decrease was evident. Expression of bFGF showed a significant decrease at week 2 in the saline-injection group from that noted in week 1, while no decrease was noted in the HA group. As for IGF, no difference was appreciable between the HA and saline-injection groups between weeks 1 and 6 (Fig. 4).

DISCUSSION

The avian shoulder is nonweight-bearing joint, and its supracoracoid tendon faces the bursa-like structure superiorly, and the shoulder joint capsule inferiorly as human supraspinatus tendon. Therefore, avian shoulder has been chosen to use in our studies.[9]

Uhthoff and Sarkar[24] suggested a link between a reparative response in the subacrominal bursa and tendon reconstitution and remodeling on the chronic human rotator cuff tear. As chronic tendon tear model is difficult to create on the animal model, we had to use acute model of tendon tear. Similar to the chronic human rotator cuff tear, we identified a reparative process ascribable to cell proliferation on the bursal aspect in our avian experimental acute model;[9] clearly, participation of cells from the bursal aspect was important in healing of this

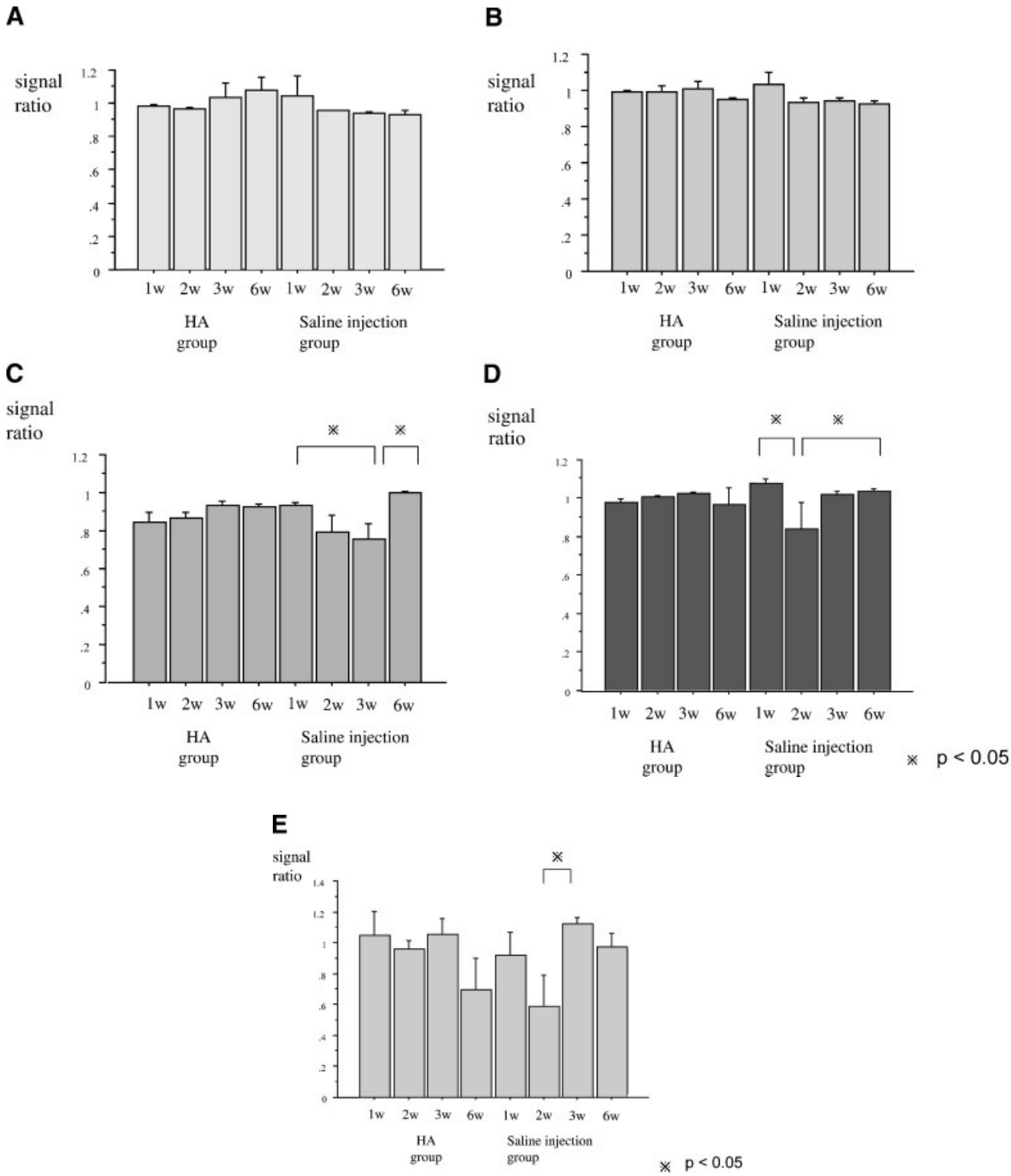

Figure 4. Expression of mRNA by real-time RT-PCR. (A) α I (I) procollagen; (B) α I (III) procollagen; (C) TGF-β1; (D) bFGF; (E) IGF.

tendon. Substances such as α I (I) procollagen, α I (III) procollagen, TGF-β, bFGF, and IGF have been implicated in the healing process.[13–16] The α I (I) and α I (III) procollagens are end-products of the tendon and peritendonous tissues.[10,25] Intratendinous administration of IGF in vivo and in vitro has been reported to stimulate collagen synthesis in the epitenon,[16] and also to enhance tendon healing.[14] TGF-β has shown to increase collagen synthesis in tendon cells,[15] while bFGF has been reported to

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increase collagen synthesis in vivo as well as cell proliferation in the damaged tendon.[13] In our current study employing a similar experimental animal model, we used gene expression for these markers of connective tissue healing to assess the effect of locally injected HA on repair of an experimental acute rotator cuff injury.

Histologically, continuity of the tendon occurred at an earlier stage in the process of healing in the HA group than in the saline-injection group. In situ hybridization concerning α I (I) procollagen at week 2 showed higher PSC expression in the superficial layer of the distal tendon with a somewhat larger increase in PSC expression in the tendon proximal to the lesion and in the superficial and middle layers of the distal part of the tendon than seen in the saline-injection group. Compared to the saline-injection group at week 3, PSC for α I (III) procollagen were concentrated specifically in the superficial and middle layers of the intervening tissue in the HA group. HA administration caused a change in distribution from saline-injected specimens, producing more expression in areas adjacent to the tendon tear. Because these changes occurred during early stages after single-dose HA administration, use of multiple HA doses might intensify them.

We have obtained the apparent loss of PSC for α I(I) procollagen at week 1 in both saline-injection and HA groups, and those for α I(III) at week 1 in both groups and at week 6 in HA group. During these ISH experiments, positive controls always showed PSC and also PSC for α I(I) at week 6 was observed. When we think about these evidences, the specimens at week 1 and 6 in both groups surely preserved the useful mRNAs, and the labeled probes for α I(I) and α I(III) procollagen mRNAs did work. In our RT-PCR study, no significant difference was observed in expression of α I(I) and α I(III) procollagen mRNAs in saline-injection and HA groups from week 1 to 6. Actually, the ISH study is not quantitative and the RT-PCR study is not truly quantitative. When we take account of our results and the accuracy of ISH and RT-PCR studies together, the real mRNA levels of α I(I) procollagen and α I(III) procollagen at week 1 in both groups and those of α I(III) procollagen at week 6 in the HA group could be lower than those of other groups. The real quantitative study is expected to be done in the future on the specimens containing small amount of mRNAs.

The main biochemical constituent of the rotator cuff is α I (I) collagen; a smaller amount of α I (III) collagen also is present. In addition to their functions in the uninjured states, these two components contribute to the process of healing. Frank et al.[26] reported that α I (III) collagen was abundant in ligaments with scar formation; the ligament scar was found to be structurally, biochemically, and mechanically abnormal even in long-term observations. Further, Riley et al.[27] indicated that amount of α I (III) collagen increased as a result of degeneration and laceration in the human rotator cuff, and that a long-term excess of this collagen species in the human supraspinatus tendon might have unfavorable biomechanical consequences. Based on the results of quantitative PCR assays in the current study, administration of HA did not increase production of a I (III) collagen, that is, HA treatment did not incite excessive scar formation in the acutely injured tendon.

Injection of HA into the subacromial bursa is performed clinically. Postulated biomechanical benefits include decreasing mechanical irritation via increased viscosity; decreasing tendon adhesion formation; and relieving impingement within the shoulder joint.[28] With respect to the articular cartilage, HA has been reported to inhibit release of inflammatory cytokines and to increase prostaglandin synthesis.[29] HA has been shown to reduce pathologic changes in articular fluid.[29]

Gomes et al.[30] reported HA promoted migration but not proliferation on human corneal epithelial cell in vitro. They suggested the beneficial effect of HA in corneal wound healing was likely to be related to rapid migration of cells. Moreover, Maniwa et al.[31] reported the velocity of synovial cell migration was increased by HA in their monolayer culture of synovial tissue derived from rabbit knee joint.

As for the effects of HA on the tendon, Wiig and Abrahamsson[8] reported that HA inhibited tendon cell proliferation, and also that HA did not enhance intratendon collagen synthesis in in vivo–in vitro combined study on the rabbit deep flexor tendons.

In our quantitative PCR study, HA did not inhibit production of TGF-β1 and bFGF mRNAs associated with tendon repair, eventually did not inhibit production of α I (I) procollagen and α I (III) procollagen mRNAs. As a result, administration of HA affected the distribution of procollagen gene expression, with cells expressing these mRNAs localized relatively specifically in the superficial layer, which was the injected side, in the vicinity of the tendon tear. Histologically HA accelerated the maturation of fibroblasts and enhanced restoration of continuity, and it appeared to promote tendon repair in the early stage of the lacerated tendon.

HA administration, which enhances gliding properties[3–6] and the decreasing inflammatory

response[2] possibly promoting tendon healing in the early stage, was considered useful, especially on the operation basis. Favorable effect on tendon healing is anticipated with use of multiple doses.

In summary, a single injection of HA inhibited the significant decreases in TGF-β1 and bFGF expressions in early tendon repair, and caused earlier restoration of continuity at the lacerated site of the tendon.

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