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Gap Junctions Regulate Responses of Tendon Cells Ex Vivo to Mechanical Loading

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Avian digital flexor tendons were used with a device to apply load ex vivo to study the effects on deoxyribonucleic acid and collagen synthesis when cell to cell communication is blocked. Flexor digitorum profundus tendons from the middle toe of 52-day-old White Leghorn chickens were excised and used as nonloaded controls, or clamped in the jaws of a displacement controlled tissue loading device and mechanically loaded for 3 days at a nominal 0.65% elongation at 1 Hz for 8 hours per day with 16 hours rest. Tendon samples were radiolabeled during the last 16 hours with ^{3}H-thymidine to monitor deoxyribonucleic acid synthesis or with ^{3}H-proline to radiolabel newly synthesized collagen. Cyclic loading of whole avian flexor tendons stimulated deoxyribonucleic acid and collagen synthesis, which could be blocked with octanol, a reversible gap junction blocker. Cells from human digital flexor tendon were used to populate a rectangular, three-dimensional, porous, polyester foam that could be deformed cyclically in vitro. Together, these results support the hypothesis that tendon cells must communicate to sustain growth and matrix expression and that an engineered three-dimensional construct can be used to study responses to mechanical load in vitro.

List of Abbreviations Used

cDNA	Complementary DNA
DNA	Deoxyriboenucleic acid
FAK	Focal adhension kinase
IGF-I	Insulin growth factor I
IP_3	3,4,5-inositoltrisphosphate
MRNA	Messenger ribonucleic acid
PCA	Perchloric acid
PDGF	BB-platelet-derived growth factor homodimer BB.
TCA	Trichloroacetic acid

Glossary

Connexin-43 a 43 kilodalton gap junction protein.

C-src tyrosine kinase the cellular version of the src oncogene that has tyrosine kinase activity.

G_0 the phase of the cell cycle between mitosis and the first growth phase of the cell cycle, G_1.

Paxillin a 67 kd protein that binds to vinculin and c-src (c-src, the cellular form of src).

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Phosphorylated src the phosphorylated form of src, a protein-tyrosine dinase first desribed as an oncogene in a Rouse sarcoma virus-infected avian muscle (sarcoma-src).

S phase a phase of the mitotic cell cycle in which DNA is duplicated.

Trauma or surgery to a flexor tendon may disrupt matrix, blood vessels, nerves and cell connections to matrix and each other. Activities that involve working with limbs above the heart or under conditions that lead to poor perfusion of a tendon also may be deleterious to tissue homeostasis.[1,3,57] Repetitive limb motion may result in injury whose etiology may involve a combination of reduced perfusion, matrix disruption, inflammation, matrix degeneration, and cell death.[1,4,31,63] Physical therapy after injury applies directed mechanical loading along the principle strain direction in a tendon and is important to achieve a satisfactory healing result.[28,69,70] It has been suggested that a therapeutic form of cyclic motion to an affected limb may assist diffusion of nutrients, growth factors from clot and second messengers to cells whose vascular supply or normal mode of diffusion has been compromised by injury.[39] However, ex vivo measurements of glucose diffusion in loaded tendons have not revealed a significant increase in response to load.[29,30]

Tendons are fibrous connective tissues designed to transmit the force of muscle contraction to bone to effect limb movement. They have a complex architecture: tendon is comprised of highly aligned matrix containing Type I collagen to provide tensile strength, elastin yielding compliance and elasticity, proteoglycans as pulse dampeners, and lipids, whose presence in the tendon epitenon may reduce shear stress induced friction.[7,19,42,45,61,64] At least two cell populations are represented in the major compartments of tendon: the surface epitenon contains large, polygonal cells whereas the internal fibroblasts are within the tendon.[7,50] The tendon surface cells reside in syncytia embedded in a fibronectin, lipid, and proteoglycan rich matrix containing Types I and III collagens, whereas the tendon internal fibroblasts are more internal in syncytial layers amidst linear and branching collagen fascicles and bundles.[6,7,27,50,52,53,61]

Tendons in running horses may be subjected to strains in excess of 0.12 (12% elongation) and strain rates of 200% per second.[32,65,66] Normal strains in tendon have been measured between a fraction of a percent to 5%.[2,68] Hannafin and coworkers[30] used 0.5% strain for 2 hours per day for as many as 4 weeks in vitro in whole canine flexor digitorum profundus tendons. In these experiments, a native phenotype and material properties in the tendon were maintained by mechanical strain. Tendon cells also responded to load by instantly releasing intracellular calcium stores, altering their cytoplasmic filament organization and content, polymerizing actin and altering their protein expression, inducing expression of novel genes.[9–11,15,23,34]

Cells must be able to coordinate their responses to environmental conditions in a wound to achieve a proper healing response resulting in orderly cell division and matrix expression.[8,15,21,41] Cells coordinate their responses to mechanical load by communicating via gap junctions.[5,41] A gap junction is a group of ion channels through which molecules of less than 1000 molecular weight pass. One level of signaling involves an increase in intracellular calcium in mechanically stimulated cells, whose wave is thought to be propagated from cell to cell by IP_3 passage through gap junctions.[18] Results of one study have shown that cells in the epitenon and internal compartment of whole tendon are connected physically to each other and express gap junctions.[41] The working hypothesis is that tendon cells must be interconnected and able to signal through gap junctions to process and respond to mechanical load signals to increase cell division and matrix expression.

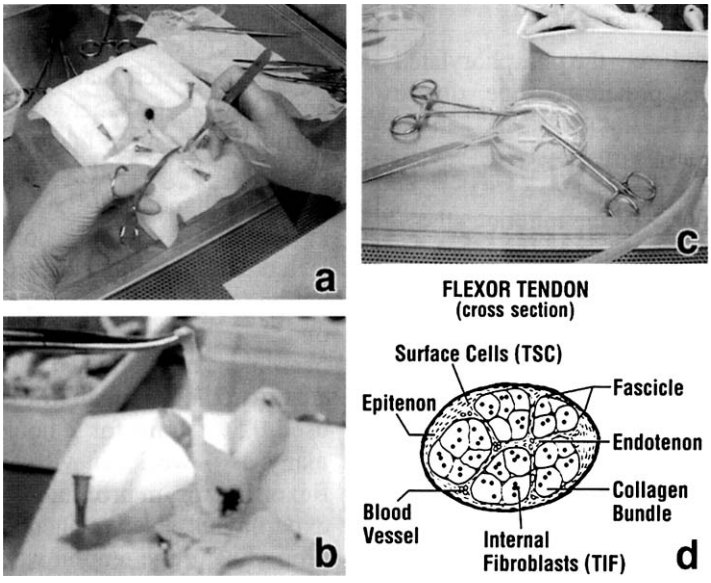

Fig 1A–D. (A) A flexor digitorum profundus tendon clamped distally, cut to release it from bone, and excised proximally is shown. (B) The tendon clamped at the distal end with hemostats, separated from surrounding tissue just before excision is shown. (C) Removal and placement with other tendons in a culture dish with phosphate buffered saline. (D) A tendon with fascicles showing the epitenon bearing the surface cells and the internal compartment, bearing the tendon internal fibroblasts is shown.

METHODS

Tendon Isolation

Flexor digitorum profundus tendons (approximately 5 cm long) from the middle claw of 52–day-old White Leghorn chickens were isolated in the following manner: 177 feet were excised at the poultry processing plant (Golden Poultry Inc, Sanford, NC), placed in plastic bags on ice and taken immediately to the laboratory (Fig 1). Feet were washed with warm water and soap, rinsed, wiped with 95% ethanol, and placed on a sterile gauze pad (Fig 1A). A foot was immobilized with rubber bands on a 5 × 10 × ½ inch board washed with ethanol; then the skin along the middle digit was cut using a scalpel fitted with a number 10 gauge blade. The skin was excised; then the fibrous tissue overlying the tendon was opened and excised free of the digit. The flexor digitorum profundus tendon was grasped at the distal end with a hemostat, were severed distally, the vinculae and extratendinous tissue were severed and the tendon was severed proximally (Fig 1B). It was important to grasp the tendons only at the ends during isolation and clamping; otherwise, artefactual mechanical stimulation of the tendon cells in the central portion of the tendon might occur. It also was important to prevent tendon dessication during the clamping procedure by maintaining the tendons in culture fluid during clamping. Tendons from 14 digits per experiment were collected and transferred to a culture dish (Fig 1C) containing Dulbecco's Modified Eagle's Medium with high glucose (4 g/L) (Gibco BRL, Grand Island, NY), 20 mmol HEPES (N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid) pH 7.2 (Sigma Chemical Co., St. Louis, MO), with antibiotics (per milliliter, 100 units sodium penicillin G, 100 μg streptomycin sulfate, and 2 μg Fungisone™ (Sigma Chemical Co.). Cells from human flexor digitorum profundus tendon were isolated using the same procedure except Medium 199 was used as the basal

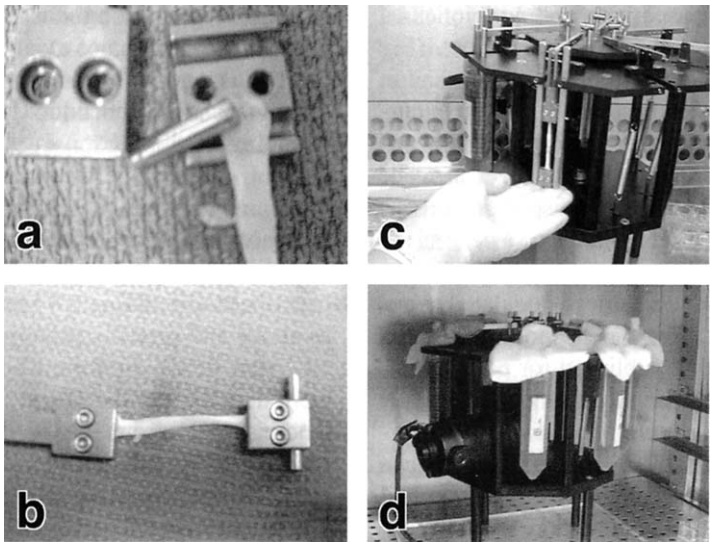

Fig 2A–D. (A) A flexor digitorum profundus tendon contacting the lower clamp face of the bottom set of jaws for (B) specimen holding for the tendon loading device. The bar across the tendon fits into the semicircular channel under the tendon and protrudes from each jaw edge so that it may be held by two arms that fit into the tendon loading device body. The other half of the jaw fits on top of the tendon and bar and is screwed together with two stainless steel screws. (C) The flexor digitorum profundus tendon clamped in top and bottom jaws and inserted in a loading frame of the tendon loading device is shown. (D) The tendons loaded in the tendon loading device are shown but the tendon loading frame and tendon are immersed in culture medium. The cotton gauze at the top covers the tube top, is immobilized with a rubber band and prevents exposure to contaminated material.

medium. Figure 1D shows the location of cells from the epitenon and internal compartment of a typical flexor tendon in cross section.

To test the concept that tendon cells can populate a three–dimensional material that can act as a tendon mimetic, human tendon cells from the internal compartment were seeded into each of six polyester foam constructs (35 mm × 8 mm × 2 mm) bonded at each end with an adhesive to the rubber membrane bottoms of a Bioflex™ culture plate. Each construct was seeded with approximately 10^6 human tendon internal fibroblasts by applying a rubber dike on each side of the construct to hold the cells and medium until the cells adhered to the foam matrix. After cell adherence and growth for 1 week, the Bioflex™ culture plate then was placed on a gasketed baseplate in a Flexercell™ Strain Unit (Flexcell International Corporation, McKeesport, PA) and subjected to a mechanical loading regimen of 1 Hz, 0.65% strain, 8 hours active, and 16 hours rest for 3 weeks. A specially designed loading post was used to apply uniaxial elongation to the membrane and construct (arctangle loading post, which is a circle with east and west sectors removed to allow the membrane and three-dimensional construct to be deformed downward at these poles; Flexcell International Corp.).

Mechanical Loading of Tendons in a Tendon Loading Device

To apply mechanical load to ex vivo tendon pieces, a seven station, minitensile testing apparatus was constructed (Fig 2). Tendon ends were clamped in specially designed, nonslip serpentine jaws (Fig 2A–B). Jaws were clamped on the tendon specimen at a gauge length of exactly 40 mm grip to grip. Clamped specimens were assembled in loading frames and placed vertically in Dulbecco's Modified Eagle's Medium containing, 20 mmol HEPES, pH 7.2, 10% fetal calf

serum, 0.5 mmol ascorbate and antibiotics as above, in 50 mL conical culture tubes (Fig 2C–D). A lever arm at the top of the tendon loading device was adjusted to 0.24 mm excursion. Excursion was controlled by the lateral position of the fulcrum under the lever arm for each station so that an exact displacement was applied. The load required to place a peak strain equivalent to 5% elongation on a group of seven tendons of 2 mm diameter was calculated as approximately 142 g/mm^2 per tendon. This is a value similar to that published for porcine tendons having an elastic modulus of 0.13 gPa.[56] However, to apply more physiologic load, displacement was set at 0.24 mm at the lever arm of the tendon loading device, yielding a nominal 0.65% elongation when a tendon gauge length of 40 mm was used. Tendons were loaded cyclically for 5 minutes to allow initial creep and load relaxation to reach a steady state, then clamps were readjusted to remove visible slack in the tendon.[35] Tendons were loaded at a nominal 0.65% elongation at 1 Hz for 8 hours of load followed by 16 hours rest for all experiments done during a 3 day period. Control, nonloaded tendons were clamped at either end but were not subjected to mechanical loading. Otherwise, control tendons were treated in the same manner as were the mechanically loaded tendons and were subjected to similar fluid movements. Tendon ends clamped in the loading jig were not included in sample evaluations.

Double Notch Wound Model

A double notch wound was created longitudinally in another group of flexor digitorum profundus tendons. (five groups, seven tendons per group, performed twice, total 70 tendons). Notch wounds approximately 4 cm long by ¼ the width of the tendon on each side (approximately 4 m × 1 mm notch wound on each side of the tendon, equaling a double notch wound) were made in one group of seven samples (control, Fig 3; double notch wound, Fig 3). These tendons were clamped in loading frames as above and subjected to 3 days of a loading regime consisting of 1 Hz, nominal 0.65% elongation for 8 hours followed by 16 hours rest (after correcting for tendon creep as above). Dimensions of avian flexor digitorum profundus tendons were approximately 5 cm in length × 4 mm in width (5 cm allowed for clamping of the tendon in the jaws). Tendon samples that were neither wounded nor loaded, but clamped at either end, and those that were only notch wounded and clamped, were transferred to loading frames and suspended in Dulbecco's Modified Eagle's Medium with serum as above and served as controls.

Avian flexor digitorum profundus tendons were divided into the following groups (seven tendons per group, experiment performed twice, total 70 tendons: (1) clamped in the tissue loading device capable of delivering displacement controlled tension to tendons ex vivo 1 Hz, nominal 0.65% elongation, 8 hours per day, 16 hours rest, for 3 days); (2) wounded

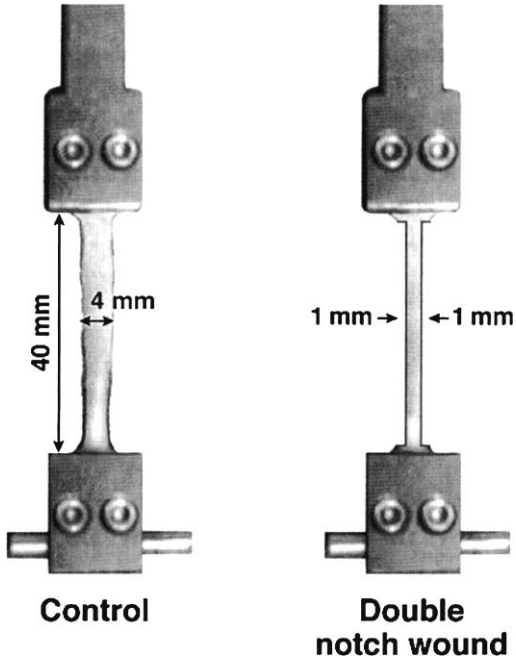

Fig 3. Shows the avian flexor digitorum profundus tendons clamped in the jaws of the loading device as a control and as a double notch wounded tendon. The wounds were approximately 50% of the total tendon width, 25% of the tendon matrix removed per side.

with a double notch wound made by cutting tissue (40 × 25 mm) from each side of the tendon; (3) wounded as in (2) above and treated with 2 mmol octanol; (4) wounded and loaded as in (1) above; (5) wounded, loaded, and immersed in 2 mmol octanol; and (6) not wounded or loaded. The tendons in each group were incubated in 45 mL of Dulbecco's Modified Eagle's Medium and 10% fetal calf serum, 20 mmol HEPES pH 7.2, antibiotics, and 0.5 mmol ascorbate. Experiments were repeated twice.

Radioactive Labeling of Tendons for DNA Determinations

Tendons were removed from the loading frames, clamped ends were excised and discarded from sampling and the remainder of the tendon samples were labeled with 0.5 μCi ^{3}H-thymidine per milliliter in 2 mL Dulbecco's Modified Eagle's Medium without serum, and antibiotics, 20 mmol HEPES pH 7.2, and 0.5 mmol ascorbate for the last 16 hours of the experiment (no load and load groups, seven tendons per group, performed three times, total 42 tendons). After incubation, samples were prepared for quantitation of DNA synthesis. Tendon samples were aspirated, washed extensively in 5% TCA to remove unincorporated radiolabel, dried, weighed and hydrolyzed in 2 N PCA. Radioactivity in duplicate portions of the PCA supernatant fluid was determined by scintillation counting. Data were expressed as ^{3}H disintegrations per minute per milligram dry weight.[8]

In an octanol washout experiment, tendons were stimulated with serum containing medium to initiate DNA synthesis in a control group. Two other groups received 2 mmol octanol to block cell communication at 24 and 48 hours, media were changed to remove octanol and fresh, serum containing medium was added. Tendons were labeled with ^{3}H-thymidine as above for the final 16 hours of the experiment, then processed to determine radioactivity in the samples (three groups, seven tendons per group, performed once). In separate experiments, tendons (n = 4 per group, four groups, 16 tendons total) were loaded as above or not loaded, labeled with ^{3}H-thymidine as above, but then fixed in 2% neutral buffered formalin at room temperature, washed extensively in neutral buffered formalin fixative, then processed for histology, autoradiography with Nuclear Track Beta emulsion (Eastman Kodak Chemical Co, Rochester, NY) and staining with hematoxylin and eosin. Radioactive nuclei were enumerated per field for the epitenon and internal compartment of tendons. An Olympus BH-2 microscope (Opelco Optical Elements Corporation, Sterling, VA) equipped with a 40 × objective lens and reticle with grid was used to perform nuclear counts. Two hundred to 500 nuclei per samples were counted per specimen and the number of nuclei bearing silver grains over the nucleus was expressed per total nuclei counted.

Radioactive Labeling of Tendons for Hydroxyproline Determinations

Tendons were removed from the loading frames, clamped ends were excised and discarded from sampling and the remainder of the tendon samples was labeled with 20 μCi ^{3}H-thymidine per milliliter in 2 mL Dulbecco's Modified Eagle's Medium without serum, with antibiotics, 20 mmol HEPES pH 7.2, and 0.5 mmol ascorbate for the last 16 hours of the 72 hours loading regime (two groups, seven tendons per group, performed twice, total 28 tendons). Samples were washed as above in TCA, dried, weighed, then hydrolyzed in 6 N HCl at 106° C for 24 hours. The hydrolyzates were dried, samples were reconstituted in diethylpyrocarbonate treated water and duplicate portions were assayed for hydroxyproline after chloramine T oxidation to the pyrrole and extraction into toluene.[47] Radioactivity in hydroxyproline was determined by scintillation counting and data were expressed as disintegrations per minute per milligram tissue dry weight.

Statistics

A SigmaStat software package (Jandel Scientific Software, San Rafael, CA) was used to apply statistical treatment to data to define significance levels. A result was deemed signifi-

TABLE 1. Nuclear Labeling Indices for Avian Flexor Digitorum Profundus Tendons Subjected to Load or Not Loaded

Tendon Location	No Load Nuclei Labeled Per Total Nuclei	Load Nuclei Labeled Per Total Nuclei
Epitenon	18 of 447, 0 of 203, 10 or 309, 0 of 232, 0 of 296, 0 of 379, 6 of 433, 6 of 220 Labeling Index = 1.59% not significantly different from the value for the epitenon	18 of 387, 16 of 502, 33 of 369, 30 of 349, 12 of 117, 14 of 382, 72 of 377, 19 of 245, 35 of 456, 32 of 258, in the internal group 29 of 263, 18 of 281 Labeling Index: 8.23% $p < 0.05$ compared with no load group
Internal Compartment	0 of 523, 15 of 565, 4 of 349, 0 of 260, 0 of 222, 0 of 306, 2 of 340, 8 of 354 Labeling Index: 0.99%	27 of 338, 1 of 404, 35 of 325, 8 of 321, 24 of 226, 8 of 228, 10 of 384 Labeling Index: 5.08% $p < 0.027$ compared with no load group

cant if $p < 0.05$ using analysis of variance (ANOVA) and a Fisher's exact test. For data in Table 1, a nonparametric procedure, the Wilcoxon rank sum test, was used in conjunction with a Kruskal-Wallis Test (chi square approximation).

RESULTS

Data in Figure 4 show that a regimen of a nominal 0.65% elongation at 1 Hz for 8 hours per day, 16 hours rest for 3 days increased DNA synthesis 4.7–fold (disintegrations per minute ^{3}H-thymidine per milligram dry weight; $n = 7$ per group, $p < 0.001$) in avian flexor digitorum profundus tendons from the great toe of 52–day-old White Leghorn chickens tested ex vivo. Treating tendons with a gap junction blocker, 2 mmol octanol, ablated the load induced DNA synthesis (Fig 4).

Table 1 shows the data for individual samples from two experiments combined (labeled nuclei divided by the total number of nuclei for each group). Data also are presented as the percent of cell nuclei labeled with ^{3}H-thymidine in the surface and deep layers of avian flexor tendon, at the anatomic midpoint of the tendon (Zone II).[60] The nuclear labeling index is the percentage of nuclei bearing developed silver grains associated with the nucleus compared with total nuclei counted. Groups included flexor digitorum profundus tendons that were not loaded or were loaded mechanically for 3 days (8 hours load, 1 Hz, 0.65% elongation, 16 hours rest per day, 3 days load). In the nonloaded tendon, surface cells of the

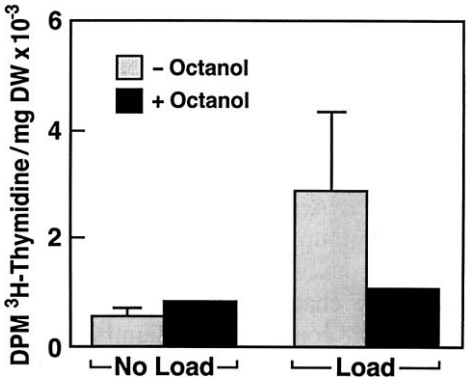

Fig 4. Cells in avian tendon increase DNA synthesis in response to a cyclic load regimen of 1 Hz, 0.65% elongation, 8 hours load, 16 hours rest for 3 days. The gap junction blocker, octanol, inhibited the response. DPM=disintegrations per minute; DW=dry weight.

epitenon had a labeling index of 1.59% (not significantly different from the value for the epitenon in the internal tendon, nonloaded group). The cells deeper in the nonloaded tendon had a labeling index of 0.99%. Cells in tendons subjected to load had a greater labeling index: epitenon cells had 8.23% labeled nuclei ($p < 0.05$ compared with the nonloaded group) and cells in the internal compartment had 5.08% of the nuclei labeled ($p < 0.027$ compared with the nonloaded group) (Table 1). Overall, cells in loaded tendons had significantly more nuclei radiolabeled with ^{3}H-thymidine than did cells in nonloaded tendons. Data are shown in the table as labeled nuclei per total nuclei. The epitenon is the surface region of the tendon. The internal compartment constituted the region of tendon between the epitenon layers, that was most internal in tendon. Not significant indicates that the labeling index for the epitenon in the no load group was not significantly different from that of the internal compartment of the no load group.

Figure 5 shows representative histologic sections of avian flexor digitorum profundus tendons prepared for autoradiography followed by hematoxylin and eosin staining in the no load and load groups. Pictures were taken at

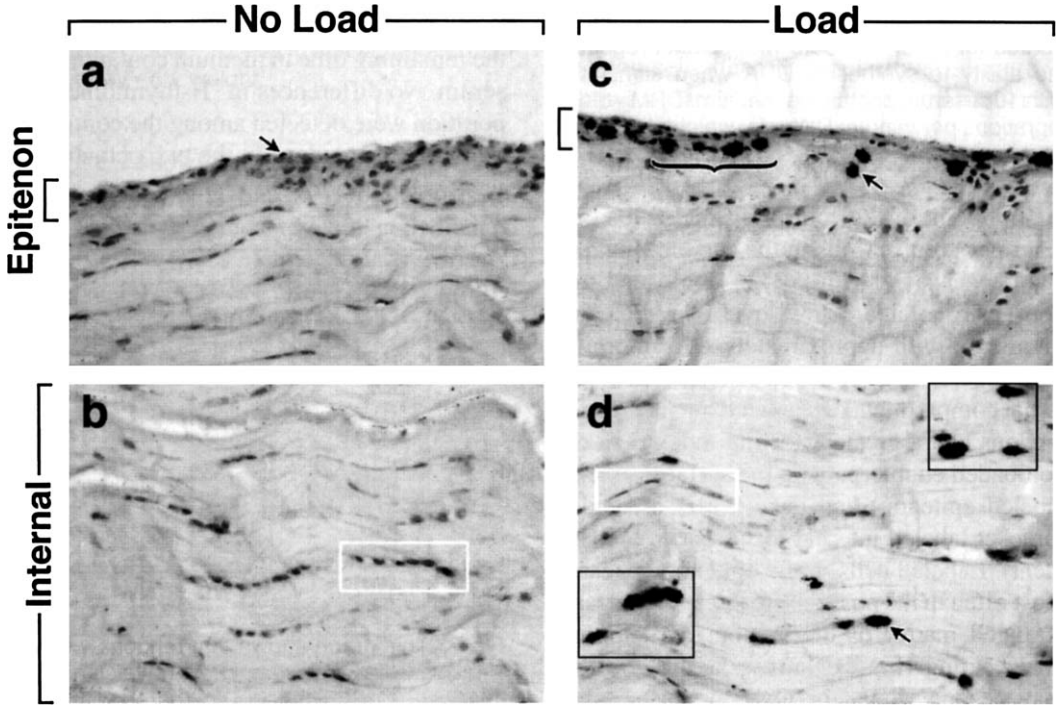

Fig 5A–D. Autoradiographs show the location and relative number of ^{3}H-thymidine-labeled nuclei in the epitenon and internal compartment of control and mechanically loaded avian flexor digitorum profundus tendons. (A) Epitenon and (B) internal compartments of nonloaded tendons incubated in vitro for 3 days. (C) Epitenon and (D) internal compartments for tendons that were loaded for 3 days at 1 Hz, 0.65% elongation 8 hours load, and 16 hours rest per day. Arrows indicate nuclei that bear exposed and developed Kodak Nuclear Track Beta emulsion as silver grains indicating incorporation of radioactive thymidine. The white boxes indicated nonlabeled nuclei. Loaded tendon cells have significantly more labeled nuclei than do control counterparts that were not loaded. Labeled nuclei in loaded tendon cells have groups of cells in close proximity synthesizing DNA (black boxes, Fig 5D), indicating that cells may have received and responded to a mitogenic signal simultaneously. (Stain, Hematoxylin and eosin; magnification, × 100).

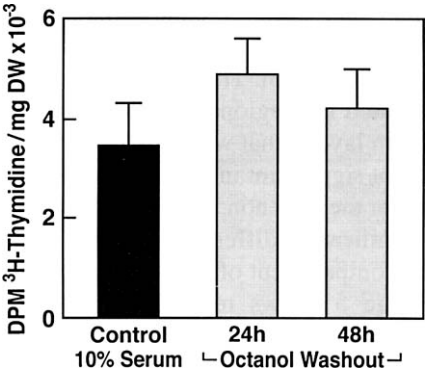

Fig 6. This graph shows that cells in tendons treated with octanol recover from the gap junction blockade by incorporating radioactive thymidine into DNA to the same extent as control tendons that were not treated with octanol. Tendons treated for 24 or 48 hours with octanol regained the ability to synthesize DNA when stimulated with 10% serum containing medium. DPM=disintegrations per minute; DW=dry weight; h=hours.

the midpoint of the control or loaded tendons (four per group). Cells in the surface (Fig 5A) and deeper layers (Fig 5B) of nonloaded tendons incorporated a low level of radioactive thymidine with approximately 60% more labeled nuclei in the epitenon cells than in the internal compartment cells. Mechanically loaded tendons had a greater labeling index than did nonloaded counterparts in the surface (bracket labeled epitenon) and deep layers of tendon (bracket labeled internal for internal compartment). Labeled cells in the epitenon appeared most often in the position closest to the linearly arranged matrix of the tendon that supports load bearing (Fig 5C, arrows). This location may be one where the environment sustains tensile load and shear stress, because the force of muscle contraction applies tensile force and as the tendon glides across extratendinous tissues or through sheath, it is subjected to shear stress. Cells with labeled nuclei in the internal compartment were distributed somewhat randomly throughout the collagen bundles. However, frequently, a group of labeled cells was detected that were likely within a syncytium of physically connected cells (radiolabeled nuclei within the confines of the black box, Fig 5D). One or more of these cells received and responded to a mechanical load signal simultaneously and advanced into S phase (cells incorporated ^{3}H-thymidine and had substantial silver grains over the nucleii). Other cells in the loaded and control cultures (white boxes) did not respond to serum or load by incorporating ^{3}H-thymidine. Clearly, there were cells in mechanically loaded tendons that did not respond to load by dividing.

Figure 6 shows the reversible inhibitory effect of octanol on DNA synthesis in whole tendon. Tendons were treated with octanol for 24 or 48 hours; then the octanol was washed out with two changes of serum containing medium, then the tendons were incubated for the remaining time in medium containing 10% serum. No differences in ^{3}H-thymidine incorporation were detected among the control tendons and the tendons in the two octanol treatment washout groups.

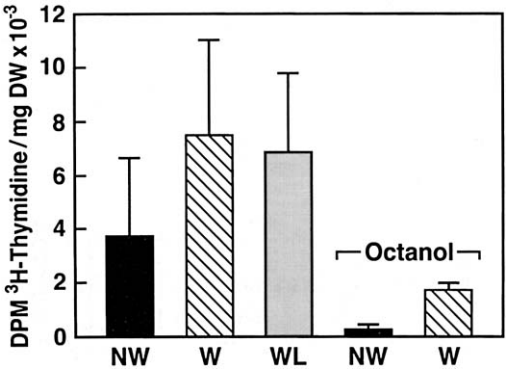

Fig 7. This graph shows that tendons wounded with a double notch wound (W) have significantly increased DNA synthesis compared with control tendons that were not wounded (NW) in the presence of serum containing medium ex vivo. Deoxyribonucleic acid synthesis in tendons subjected to mechanical loading in addition to wounding were not stimulated additionally (W compared with WL). Treatment of tendons that were not wounded (NW) or tendons that were wounded (W) with the gap junction inhibitor, octanol, ablated the increase in DNA synthesis in normal tendon and in tendons stimulated by wounding. DPM=disintegrations per minute; DW=dry weight.

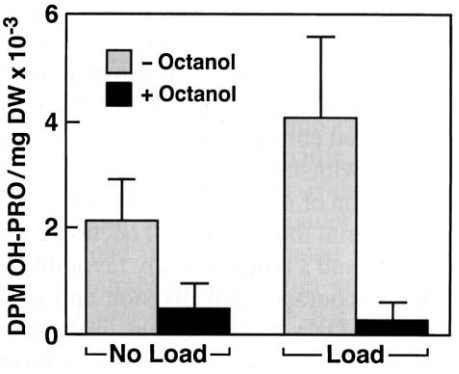

Fig 8. This graph shows that mechanical loading (1 Hz, 0.65% elongation for 8 hours followed by 16 hours rest) had increased collagen synthesis. Treatment of tendons with the gap junction inhibitor, octanol, ablated the stimulatory effect of mechanical load. DPM=disintegrations per minute; DW=dry weight.

Figure 7 shows that tendons that received a double notch wound had a twofold increase in DNA synthesis compared with nonwounded controls ($p < 0.001$, tendons that received a wound and were loaded also showed a twofold increase, ($p < 0.001$, compared with nonwounded control), but this was not significantly different from values for wounded tendons alone. Treating tendons with a gap junction blocker, 2 mmol octanol, ablated the wound induced DNA synthesis. Treating nonwounded tendons with octanol decreased DNA synthesis 3.4 fold ($p < 0.017$). Treatment of wounded tendons with octanol also reduced DNA synthesis (22% of wound control, $p < 0.004$).

Figure 8 shows that load increased collagen synthesis by 1.5–fold compared with values for the nonloaded controls ($p < 0.01$). Treating tendons with the gap junction inhibitor octanol reduced the stimulatory effect loading had on collagen synthesis ($p < 0.001$).

Figure 9 shows an embodiment of a deformable, material that can be used to support tendon cell adherence and growth. Figure 9A shows a plastic, Bioflex™ six well rubber bottom plate with each 35 mm diameter well containing a 35 mm × 8 mm × 2 mm polyester foam (brackets). Each part was bonded to the rubber membrane at its ends with an adhesive (Fig 9B, black rectangles at east and west poles). The black arrow in Figure 9B shows the principle strain direction in a well when an

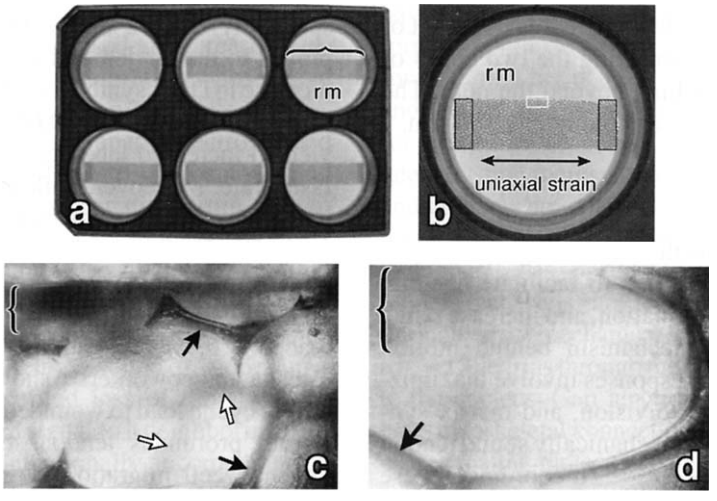

Fig 9A–D. Images in Figure 9 show (A) a Bioflex™ rubber bottom culture plate with (B) polyester foam tendon mimetics bonded to each 35–mm diameter well. rm=rubber membrane (C) Human tendon internal fibroblasts growing along a tract defined by the bracket at the upper left of the figure are shown. Cells also have populated the interstices of the matrix in the deeper regions. (D) A ×50 magnification of the material (black arrow) and cells and matrix (bracket) filling the material.

arctangular (combination of arc and rectangle) loading post is used to allow the rubber membrane to deform downward only at the east and west poles where the black rectangles demarcate the areas of adhesive bonding of the construct to the rubber membrane. The small white rectangle is the region from which images of human tendon cells were taken for Figure 9C (×25 magnification) and 9D (×50 magnification). Figure 9C shows the view at the top edge of the white enclosed region from Figure 9B. Tendon cells have grown in a dense band laterally along the outer boundary of the construct (dark bracket top left, Fig 9C). Matrix has been deposited between the cells evidenced by two observations: (1) the distance between cells is increased over that in cells grown in two–dimensional culture; and (2) the cells in the three–dimensional culture are difficult to dissagregate by use of trypsin. The upper black arrow at the 1:00 o'clock position points to one arm of the porous material and the second black arrow at 5:00 o'clock points to an arm deeper in the material (Fig 9C). The two white arrows point to arms that are even deeper in the polyester matrix. Figure 9D shows a ×50 magnification of a pore. The black arrow points to the arm of the polyester material. The black bracket indicates a tract of cells and matrix connecting the two edges of the pore and stretching beyond the pore. The average pore size is approximately 500 μm.

DISCUSSION

In patients, motion therapy strategies after tendon repair are designed to facilitate healing, reduce adhesion formation, and increase range of motion. The mechanism behind motion therapy and these responses involve maximizing cell migration, division, and matrix synthesis to yield a biomechanically sound tendon that readily glides.[26,58,60] To coordinate these responses, mechanical and chemical signals must be communicated between cells and among cells. Successful engineered tendon or ligament replacements must include designs that allow for cell to cell connectivity to allow intercellular communication. In addition, mechanical loading of tendon cells in monolayer cultures and in whole tendons can increase cell number and collagen synthesis.[12,14] Therefore, an engineered construct with sufficient tensile strength to withstand the biomechanical rigors of application of muscle force should be made with a material that favors cell occupancy and orientation and a biochemically favorable matrix that encourages cell division and matrix expression. Data in the present investigation indicated that cells in whole tendon subjected to cyclic load ex vivo required functional gap junctions to mount mitogenic or matrigenic responses. The gap junction blocker, octanol, significantly reduced the ability of cells from normal or wounded tendons to synthesize DNA or collagen in response to mechanical load. Results of washout experiments indicated that removal of octanol restored the ability of cells in tendon to incorporate radioactive thymidine into DNA.

In response to mechanical load, tendon cells deform when contacted by collagen fibrils by sustaining membrane indentations.[42] Slack and coworkers[58] showed that embryonic tendons subjected to low rates of cyclic loading (two or six cycles per minute, 0.033 and 0.1 Hz, respectively) for 72 hours with a 0.9 g mass pinned to the tendon end ex vivo) had a 50% increase in DNA synthesis compared with nonloaded controls. The 0.033 Hz frequency yielded increased labeling compared with the faster rate. Moreover, collagen synthesis also was increased in loaded tendons. Hannafin and coworkers[30] showed that adult canine flexor digitorum profundus tendons maintained morphology and water content when actively loaded at 0.5% elongation at 0.5 Hz ex vivo. Tanaka and coworkers[60] showed that application of cyclic load to wounded avian flexor digitorum profundus tendons ex vivo induced epitenon cell migration in the region of the hemisection and wound. However, the mechanisms responsible for this response have not been elucidated. Given the potential of the cytoskeleton in transducing mechanical load signals, the roles of actin and tubulin were inves-

tigated in tendon cells.[33] Actin per tubulin ratios increased in response to cyclic compression in vitro in tendon internal fibroblasts.[9] Tendon surface cells of the epitenon and tendon cells of the internal compartment phosphorylated src, FAK and paxillin, proteins of the focal adhesion complex and other unknown proteins within seconds to minutes in response to cyclic load in vitro, signaled with an increase in intracellular calcium concentration and, in synergy with PDGF-BB and IGF-I, synthesized DNA.[14,15,19] Taken together, these results show that tendon cells ex vivo in whole tendon and in vitro in culture respond to mechanical load in an organized fashion. How might these events be coordinated?

Epitenon cells and internal fibroblasts in flexor tendons in vivo are layered in longitudinal syncytia that are optimal for rapid, repeated electrical or chemical coupling similar to osteocytes in bone.[36,41] Tendon cells in vitro are coupled via gap junctions and respond to a mechanical perturbation with a micropipet stimulation of the plasma membrane by releasing intracellular calcium stores and propagating a calcium wave to adjacent cells for as many as four to seven cell diameters.[13,34] Sanderson and coworkers[55] showed that indenting a target epithelial cell membrane with a 1 μ-wide pipet tip caused an increase in intracellular calcium concentration whose wave was propagated to adjoining cells by IP_3 through gap junctions. Heparin electroporated into the cells blocked IP_3 receptors and blocked the wave propagation from cell to cell.[18] Likewise, the gap junction blockers halothane and octanol also ablated the calcium wave propagation. Charles and coworkers[22] showed that loss of connexin-43 expression, a gap junction protein, resulted in poor junctional competence and loss of the ability to transmit a calcium signal to a neighboring cell. Transfection of junctionally incompetent c6 glioma cells with connexin-43 cDNA restored intercellular communication.[22] Therefore, gap junctions represent one mechanism cells use to regulate a response to mechanical and chemical signals.

As with every other cell type tested thus far, avian tendon cells express gap junctions.[16,17,28,41,59,67] Gap junctions are localized channels in the plasma membranes of contacting cells that are 1 to 2 nm apart, comprised of subunits arranged in a hexameric pattern and pass ions and molecules less than 1000 molecular weight, such as inositol phosphates or Ca^{2+} between and among cells.[16,20,40,49,51,54] Compounds such as acetylcholine or the anesthetics, halothane, heptanol, isoflurane or octanol block the junction, probably by altering calcium homeostasis in the anesthetics and by a protein kinase C mediated mechanism with acetylcholine.[43,46,48,57] Also, the relative amounts of connexin 43 gap junction protein in a cell are important because vole NIH 3T3 cells have eight times the connexin protein and better junctional coupling than do mouse NIH 3T3 cells.[25] Upregulation of the c-src tyrosine kinase results in a reduction in cell to cell communication by increasing kinase activity and presumably phosphorylating connexin protein and decreasing the channel open state.[25] Avian cells have at least three forms of connexin 43: a 42 kd nonphosphorylated form and two intermediate forms from 44 to 47 kd that are phosphorylated at serine.[37,44] Quiescent tendon surface cells have predominantly the nonphosphorylated form of connexin 43 but have phosphorylated forms during log phase.[11] Tendon surface cells and tendon internal fibroblasts express mRNA for connexins 42, 43, 45 and 45.5 semiquantitated by polymerase chain reaction detection and cloning, but connexin 43 is the only form detected by Northern analysis.[11,62]

Ingber[33] has postulated in his tensegrity model that cells are connected and signal through direct mechanical linkage from the matrix via integrins through the cytoskeletal system to the nucleus. Gap junction proteins may not have cytoskeletal connections but are known to pass signaling molecules intercellularly and are essential for intercellular communication of strain signals.[16,38,55] Connexin 43 gap junction expression is upregulated by mechanical load in cultured tendon cells.[5] However, other study recently has shown that connexin 43 is upregu-

lated by load in vascular smooth muscle cells in culture.[24] Bennett et al[16] think that the connexins are intimately involved in regulating embryogenesis and development. Tendon cells subjected to load in whole tendons and labeled for DNA synthesis by autoradiography clearly showed groups of labeled cells rather than only randomly distributed labeled cells. This observation may indicate that specific cells in a given region received a mechanical stimulation that directed them to divide. Similar to the results of the cell deformation experiment using a micropipet and Ca^{2+} signaling, they in turn signaled their nearest neighbors to exit G_0 and enter S phase. It is clear from the results of histology and autoradiography in ^{3}H-thymidine labeling experiments that not all tendon cells connected in syncytial arrays in either the epitenon or the internal compartment were stimulated to divide by mechanical load. If one assumes that each cell actually is subjected to a similar mechanical environment, then some cells likely are inhibited from entering S phase by some specific mechanism. Communication and signaling through gap junctions is one effective way to regulate which cells receive a signal allowing them to advance through cell division and which cells do not. Therefore, it is likely that gap junction regeneration and function also are essential for an organized wound healing response in tendon, tendon grafts, or tissue engineered constructs designed for implantation in a patient, particularly in response to mechanical load. The polyester foam material used in the present study as a tendon mimetic can support human tendon cell adherence, growth and matrix production. Application of cyclic mechanical load may induce cell alignment along the principal strain direction and stimulate cell and matrix production. This material, and other materials that can support cell ingrowth and matrix expression, can be used to model tendon growth, development and replacement ex vivo.

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