

On Changes in Length of Dense Collagenous Tissues:  
Growth and Contracture

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## ABSTRACT

This paper summarizes experimental work on ligament growth and contracture carried out in our laboratories over the past decade and a half.

Although previous bone, muscle and tendon studies have shown that these tissues grow, for the most part, at "growth plates", our marking suture studies demonstrated that, in ligament, longitudinal growth and contracture both occur as diffuse processes in which material is added or removed interstitially. In our studies, growth of ligamentous tissue appeared to be influenced by systemic hormonal factors, but was locally mediated by mechanical tension, or lack of tension, which caused an increase or decrease in growth throughout the length of the ligament.

We found that the actin cytoskeleton of the fibroblast was involved in the contracture of lax ligaments, presumably producing the necessary mechanical force. This contracture phenomenon is hypothesized to retighten microstretch ligament injuries throughout life and to result in the clinical problem of contracture of capsular ligaments during joint immobilization. Simulated stress generated electrical potentials (SGEPs) diminished the contracture process, indicating that an absence of SGEPs may serve as one signal that the tissue is not being mechanically loaded.

Our work supports the hypothesis that the mechanism of length changes in ligament and tendon involves the sliding of discontinuous collagen fibrils past one another. Changes in fibril overlap during growth and contracture have been demonstrated. Small polycations have been found to markedly increase the strain in loaded rat tail tendon and to allow the extraction of intact collagen fibrils from vertebrate ligaments. These fibrils taper to amino terminal points on both ends. The polycations are presumed to interfere with putative "interfibrillar bonds" which attach one fibril to another and thus prevent fibril sliding under normal circumstances.

## INTRODUCTION

Although most orthopaedists believe that they understand how the musculoskeletal system grows, upon reflection many recognize that they only understand bone growth. Until recently, little was actually known about the mechanisms of growth of ligamentous tissues or, the seemingly related phenomenon, ligament contracture. This may be, to some extent, because there appears to be little clinical relevance to a body of knowledge concerning ligamentous growth. Certainly, complete failure to develop ligamentous structure is rare and is usually associated with clinical syndromes of such severity that the absence of a ligamentous structure is of little clinical import. However, there are several disorders of development in which inadequate or excessive ligamentous growth could result in joint deformity. Idiopathic scoliosis may be an example of such a disorder (Miller, 1982; Skogland and Miller, 1980). Nonidiopathic scoliosis is commonly found in diseases of ligamentous laxity and idiopathic scoliosis develops during the growth spurt of adolescence, a time when ligaments might grow more than their underlying joints, resulting in a state of temporary ligamentous laxity (Miller, 1982). In addition, it is possible that some disorders, such as clubfoot, often attributed to contracture, may be partially caused by inadequate ligamentous growth. Certainly, methods for inducing ligamentous growth in contracted tissues, producing contracture in lax tissues, and modifying contracture in certain conditions might be used clinically to ameliorate disease or deformity.

## LIGAMENT GROWTH

During ligamentous growth, several things must be accomplished. The ligament must be elongated in order to accommodate the increased size of the underlying joint. It must undergo an increase in its cross-sectional area in order to have the strength required by the loads produced in the larger underlying joint. A third, quite interesting, phenomenon which must be accommodated is that, in instances where the ligament inserts on the metaphysis, the original insertion site of the ligament is constantly being moved away from the joint by growth of the underlying physis. Therefore, the ligament insertion site must constantly undergo translocation on the metaphysis in order to maintain its position near the joint line. In our studies, we have examined the anatomy of longitudinal ligament growth, and the mechanisms controlling such ligament growth.

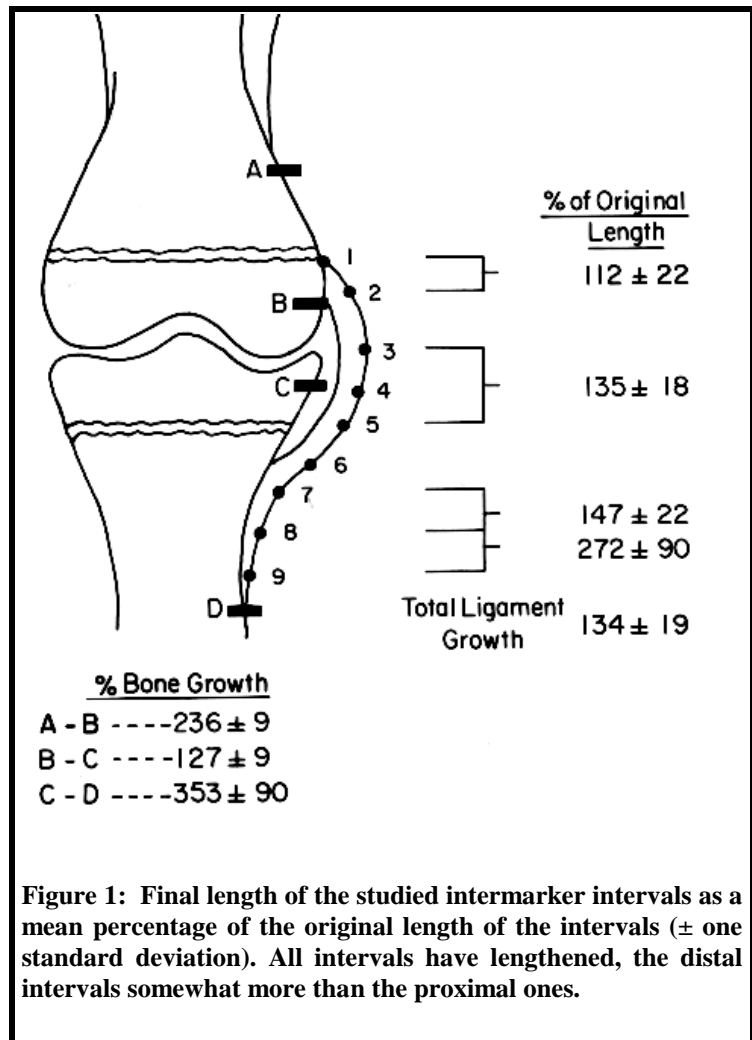
## ANATOMY OF LIGAMENT GROWTH

Other major musculoskeletal tissues grow for the most part at a localized area or "growth plate". Obviously, bone grows longitudinally at the physis. In the past, marking suture type studies have indicated that muscle and tendon both grow longitudinally by adding on new material, for the most part, at the muscle tendon junction, although some interstitial lengthening does occur (Crawford, 1950; Ziv and, Blackburn, et al 1984). In our studies, longitudinal ligament growth occurred through a process of diffuse interstitial addition of length, as demonstrated by marking sutures (Muller and Dahners, 1988; Wessels and Dahners, 1988). In these studies, marking sutures placed at intervals throughout the length of seven 6 week old (growing) rabbit medial collateral ligaments (MCL) showed increases in the intermarker intervals at each interval (Figure 1, next page).

The most distal marking sutures became incorporated in the bone of the tibial metaphysis. The intermarker interval from this "bone incorporated" suture to the next more proximal suture in the MCL rapidly enlarged at a much higher rate ( $272 \pm 90\%$ ) than the interstitial markers ( $112 \pm 22\%$  to  $147 \pm 22\%$ ), giving the impression that there is indeed a "growth plate" at the tibial insertion where rapid ligament growth occurs. However, comparison to growth at the tibial physis ( $353 \pm 90\%$ ) confirmed that the amount of growth at this "growth plate" was not even sufficient to completely translocate the insertion site of the ligament proximally on the metaphysis (to accommodate for bone growth) and that elongation of the actual body of the ligament (origin to insertion) occurred interstitially (Muller and Dahners, 1988). A similar mechanism for translocation of metaphyseal tendon insertion sites has been noted by Videman (Videman, 1970).

Studies carried out by Frank et. al. using auto-radiography in rabbit MCLs demonstrated a high rate of cell division in the region responsible for translocation of the insertion. They also noted interstitial addition of matrix during growth (Frank and Brodie et al, 1987). They observed an encapsulating "periligament" surrounding the MCL which they felt might be contributing to expansion of its cross-section by adding matrix at the surface.

We carried out a second study in the rabbit deltoid ligament (which originates on the distal tibial epiphysis at the medial malleolus and inserts on the talus and calcaneus without crossing a physis). Using seven 5 week old rabbits followed 10 weeks we demonstrated uniform increases in intermarker intervals (Table 1) without the "growth plate like" phenomenon seen in the distal MCL secondary to translocation (Wessels and Dahners, 1988).



**Figure 1:** Final length of the studied intermarker intervals as a mean percentage of the original length of the intervals ( $\pm$  one standard deviation). All intervals have lengthened, the distal intervals somewhat more than the proximal ones.

**Table 1**  
Percent of Growth of Four  
Longitudinal Ligament Intervals in  
Seven Rabbit Deltoid Ligaments

Interval	% Growth
Proximal	37 ± 9
Midproximal	36 ± 5
Middistal	34 ± 11
Distal	33 ± 19

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Values are given as means ± SE.

In summary our work described above demonstrated that longitudinal ligament growth occurs interstitially rather than at a “growth plate” or growth region. Subsequent to our work in ligaments, other investigators have restudied growth in tendons and found, in contrast to the work by Crawford in the 1950’s indicating that tendon grows at the muscle tendon junction (Crawford, 1950), that tendon, in fact, also grows interstitially throughout it’s length (Fugio and Nishijima et al, 1994; Nishijima and Yamamuro et al, 1994) indicating that tendons probably lengthen longitudinally by a mechanism similar to ligament.

#### CONTROL MECHANISMS FOR LIGAMENT GROWTH

In order for knowledge of ligament growth to be clinically relevant, an understanding of the mechanisms which control ligament growth is important. Growth of the bone physes and of other tissues in the body is to a large degree influenced by growth hormone through intermediary IGFs (insulin like growth factors or somatomedins). Bone growth, however, is influenced as well by mechanical loading, blood supply, and numerous other factors. It is reasonable to postulate that ligamentous tissue might respond to growth hormone or other systemic factors, to mechanical loading (especially that which results from underlying bone growth), and perhaps to other, as yet undetected, influences (i.e. growth factors or cytokines secreted by the nearby physes). We have attempted to evaluate the effects of some of these factors on the growth of ligamentous tissues.

In an initial series of studies, marking sutures were applied to the lateral collateral ligaments of growing rabbits (Dahners and Muller, 1988; Dahners and Sykes et al, 1989). The fibular head was separated from the proximal tibia and the fibular shaft resected. Using implanted rubber bands, longitudinal tension was applied through the freed fibular head. In six week old rabbits, this resulted in a moderate increase in the rate of elongation of the ligament intermarker distances (growth) as compared to controls in which the fibular head was not detached (Table 2, second line). A marked increase in growth was observed when these studies were performed in 4 wk old rabbits (Table 2, third line). Surprisingly, in shams (the fibular head was freed, but the rubber band was left lax and no tension was applied) the ligaments continued to elongate (Table 2, first line). It had been expected that these sham ligaments, in which the fibular head had been cut free from the proximal tibia, would undergo a contracture process (a

process discussed further in the section on contracture of ligaments) but they did not. It has been postulated that in these sham animals, the fibular head may have scarred back down to the proximal tibia quickly enough that a distraction force continued to be applied to the LCL. This is probably the case because (as discussed below), in our growth plate studies, the MCL did not continue to grow after arrest of the underlying tibial physis.

**Table 2:**  
Ligament Elongation in Growing Rabbits  
With and Without Tension

	Control (Fibular Head Still Attached)	Sham (Rubber Band Lax)	Experimental (Rubber Band Under Tension)
6-week old rabbits	12 ± 8%	16 ± 4%	NA
6-week old rabbits	14 ± 6%	NA	29 ± 11%
4-week old rabbits	79 ± 5%	NA	140 ± 18%

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Values are given as means ± SE.

This study supports the view that ligament growth proceeds at a certain rate under the influence of systemic factors, but that the rate of growth can be modified by the degree of mechanical tension applied by growth of the underlying bone. We hypothesize that ligament growth is influenced more by steady, constant tension (as applied by the rubber band in the above experiment) than by intermittent peak tension. This is substantiated clinically in that active children are not noted to have more joint laxity than inactive children, despite the high peak stresses imparted to growing ligaments by their physical play.

In an attempt to elucidate the role of growth hormone in ligament growth, we implanted LCL marker sutures with and without application of tension to the fibular head (via a rubber band) in 21 mature rabbits in order to determine whether growth hormone could cause a resumption of ligament growth in adults (Dahners and Sykes et al, 1989). Interestingly enough, neither administration of growth hormone (in quantities sufficient to produce excessive hair growth and new periosteal bone formation), nor application of tension, nor a combination of growth hormone and tension was sufficient to cause a resumption of the growth of the LCL in these mature animals (Table 3).

**Table 3**  
Ligament Elongation in Adult Rabbits  
With and Without Tension and/or Growth Hormone

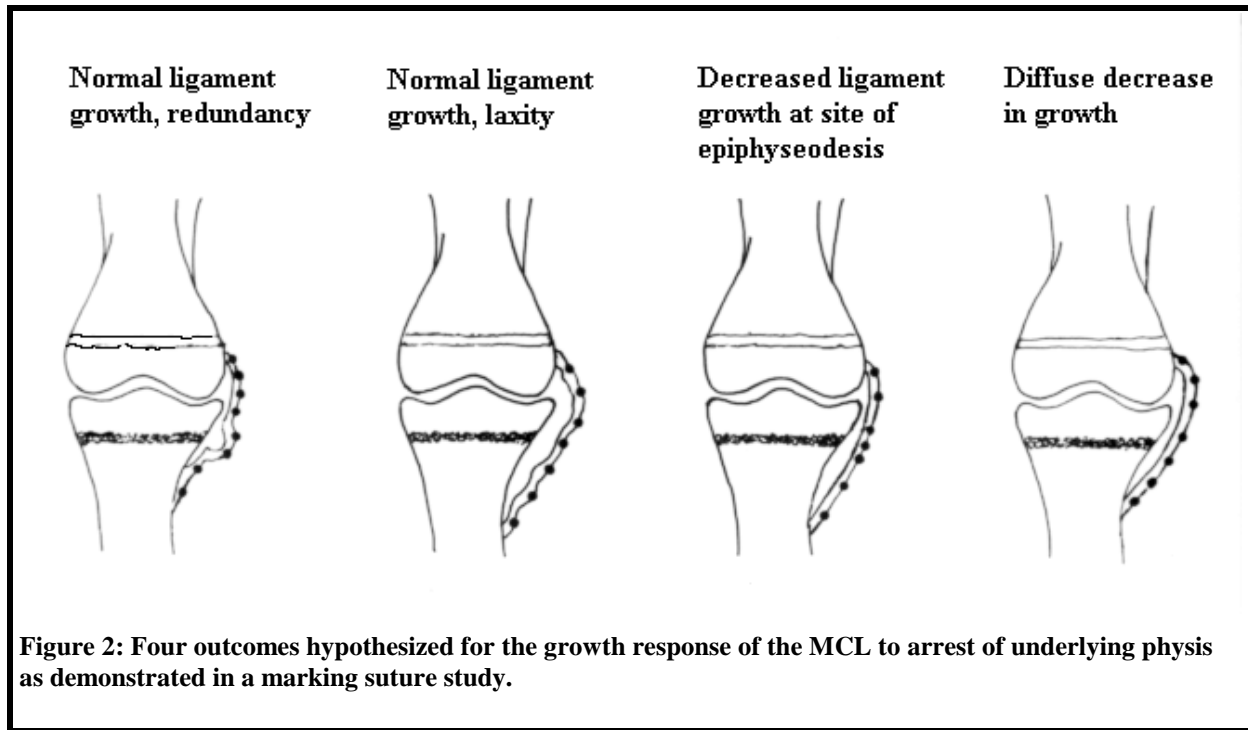
	Control (Fibular Head Still Attached)	Sham (Rubber Band Lax)	Experimental (Rubber Band Under Tension)
Adults with GH	2 ± 4%	-2 ± 5%	3 ± 7%
Adults without GH	NA	4 ± 6%	5 ± 9%

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Values are given as means ± SE.

One might wonder whether the clinical use of Ilizarov type devices to stretch out capsular ligaments in the correction of joint contractures provides evidence that with sufficient tension, growth can be induced in adult animals. As yet, it has not been determined whether the Ilizarov technique causes an actual resumption of growth or a gradual, but traumatic, stretching of the ligaments.

It appeared that something could be learned about ligament growth and the mechanisms which control it by investigating the alterations in MCL growth produced by physeal arrest of the proximal tibial physis (which the MCL crosses). Four possible outcomes were hypothesized (Figure 2): (1) If ligamentous growth is in large part under the control of a systemic factor such as growth hormone, but is to some extent predetermined (genetically or otherwise) to occur at certain locations, one might expect that physeal arrest would cause a mounding up of redundant ligament tissue directly over the arrested physis, with the remainder of the ligament growing appropriately, relative to the growth of immediately underlying structures. (2) If ligamentous growth is preprogrammed or is solely under the control of a systemic factor, the MCL would continue to grow and become generally lax. (3) If ligamentous growth is for the most part under the precise control of local factors, growth might occur normally in the remainder of the ligament, but suffer an arrest directly over the physis. (4) If ligamentous growth is for the most part controlled by mechanical tension applied to the ends of the ligament, physeal arrest might diminish interstitial growth throughout the length of the ligament. In our study of this question, carried out with intermarker sutures and curettement epiphysiodesis in fourteen 4 week old growing rabbits, we found that ligament growth was diminished diffusely at intermarker intervals throughout the length of the ligament, thus supporting the fourth hypothesis (DeGnore and Dahners, 1991).



Thus, we have made the following conclusions regarding ligament growth: (1) Elongation of ligamentous tissues occur by diffuse, interstitial addition of matrix to increase the length rather than by a "growth plate" phenomenon. (2) There is a rapid growth area for translocation of the insertion of ligaments which insert on the metaphyses of bones (i.e. the knee MCL) in order to maintain the insertion of those ligaments in their correct locations despite the movement of the underlying bone away from the joint line. (3) Growth of ligaments is influenced by the application of constant longitudinal mechanical tension to the growing ligament. (4) Growth hormone alone or in combination with added longitudinal tension was not capable of causing a resumption of ligament growth in mature animals. It may be, however, that the amount of tension applied to these mature rabbit ligaments was insufficient, and that a greater degree of tension (i.e. such as is provided clinically by Ilizarov devices) might have resulted in a resumption of growth.

### LIGAMENT CONTRACTURE

It is widely presumed that soft tissues follow some modified version of Wolff's law for the remodeling of bone. Wolff's law states that "every change in the form and the function of a bone, or in its function alone, is followed by certain definite changes in its internal architecture and secondary alterations in its external conformation". A simple translation is that "form follows function". It is assumed that form also follows function in the soft tissues, and there is certainly evidence to support that conclusion. If Wolff's law does indeed apply to ligamentous tissue, one might expect that an alteration in the mechanical loading of a ligament might result in: (1) changes in the size/strength of the ligament and the literature does provide evidence for this (Binkley and Peat, 1986; Burroughs and Dahners et al, 1990; Gomez and Ishizue et al, 1988; Hart and Dahners 1987; Lechner and Dahners, 1991; Noyes and Torvik et al, 1974; Tipton and

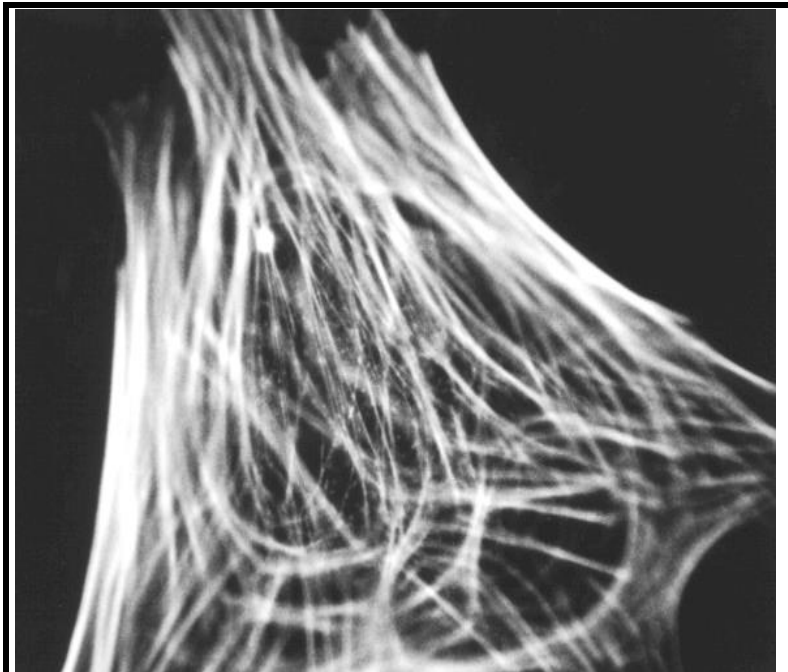


Schild et al, 1967; Vailas and Tipton et al, 1981; Woo and Gomez et al, 1987) ; (2) changes in the matrix organization/stiffness of the ligament and the literature supports this as well (Akeson, 1961; Padgett and Dahners, 1992; Dahners and Torke et al, 1989; Frank and MacFarlane et al, 1991; Schaberg and Dahners et al, 1992); or (3) changes in the tightness/laxity/length of the ligament which we will specifically address below.

It is perhaps somewhat unique to ligamentous musculoskeletal tissues that they must be of the correct length to function properly. A bone that is slightly too long or too short will usually still function well, and muscle/tendon shortening or lengthening can be compensated for by dynamic muscle contraction/elongation to some extent. Incorrect ligament length, however, results in joint dysfunction as the constraints against abnormal motion of the articular surfaces are lost with laxity and range of motion is lost with excessive tightness.

Thus one would expect that ligaments must have some means for adjusting their length. If that were not the case, traumatic injuries and even cumulative micro-stretch injuries to ligaments throughout a lifetime would result in older patients having generally more lax joints, a phenomenon which is exactly the opposite of the clinical situation. A common clinical finding is that unloaded ligaments not only atrophy, but also undergo contracture. This is most apparent in a joint immobilized in one position for an extended period of time. The relaxed capsular ligaments develop a contracture which inhibits remobilization of the joint. It would seem likely that a natural process, which tightens ligaments not subjected to loading, is responsible for both of these phenomena: the prevention of ligament laxity from cumulative micro-stretch injuries *and* the development of capsular contracture around immobilized joints.

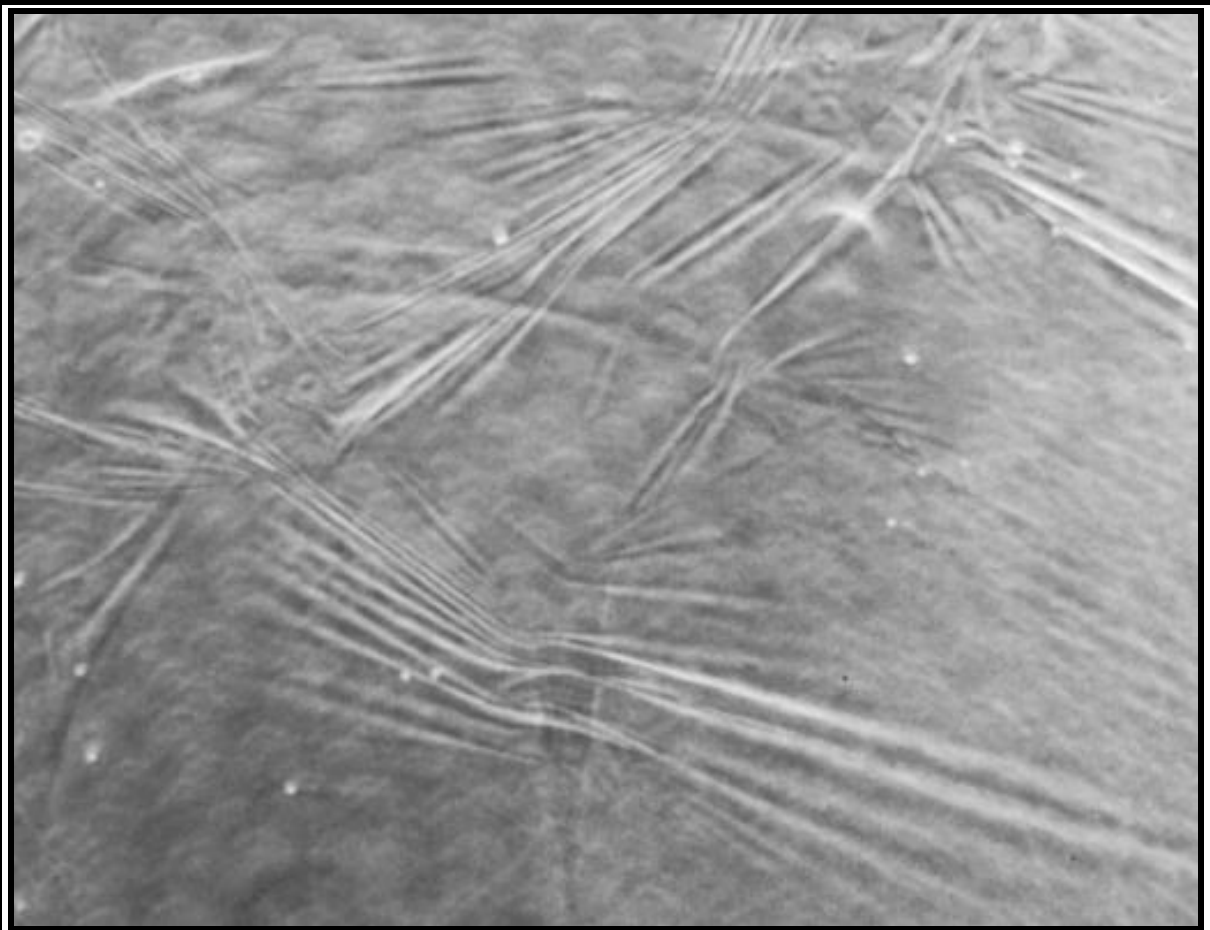
Early studies of the ligament contracture process by other investigators showed that simply inducing additional cross linking of “folded collagen fibers” in ligaments lying in a relaxed position did not cause shortening of that ligament (Peacock, 1966). Based on the discovery that actin is contained in the cytoskeleton of almost all cells in the body and is markedly increased in the myofibroblasts responsible for wound contracture, (Gabbiani and Ryan, 1971), we hypothesized that ligament fibroblasts may undergo a similar process. We found that ligament fibroblasts, when grown in tissue culture, have a marked actin cytoskeleton (Figure 3) (Dahners and Banes et al, 1986). In tissue culture, these fibroblasts are in a mechanically stress free environment, presumably similar



**Figure 3: Rat fibroblast stained with NBD Phalloidin to demonstrate its prominent actin cytoskeleton in tissue culture.**

to the low stress environment of a lax ligament (which would be expected to undergo contracture).

When grown in tissue culture on a thin silastic membrane, Harris et. al. found that embryonic fibroblasts will produce wrinkles in the membrane through the mechanical force produced by their actin cytoskeleton (Harris and Wild et al, 1980). We have subsequently reproduced this phenomenon with ligament fibroblasts from more mature animals (Figure 4), although we have not previously published these photographs.



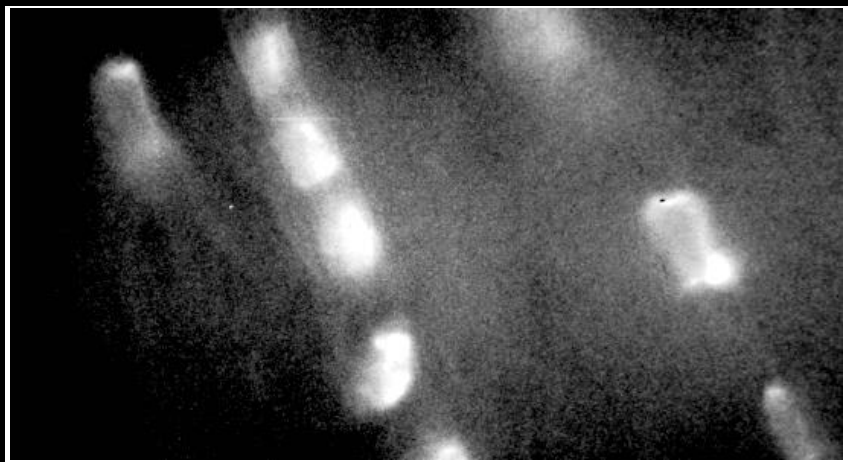
**Figure 4: Rat fibroblasts grown on a thin silastic membrane. Note that they are producing wrinkles in the underlying silastic.**

In our experiments done with surgically Z lengthened rat MCLs (which undergo a contracture process that restores normal length) and in rat ACLs undergoing contracture during joint immobilization (Figures 5a and 5b), staining for actin was enhanced, indicating that fibroblasts in both of these contracture processes have increased their actin cytoskeleton (Dahners and Banes et al, 1986; Wilson and Dahners et al, 1988). The contractures in the Z-lengthened ligaments occurred diffusely throughout the ligament (based on marking suture study) rather than in any particular area. This ACL contracture process is in contrast to the clinically lax anterior cruciate

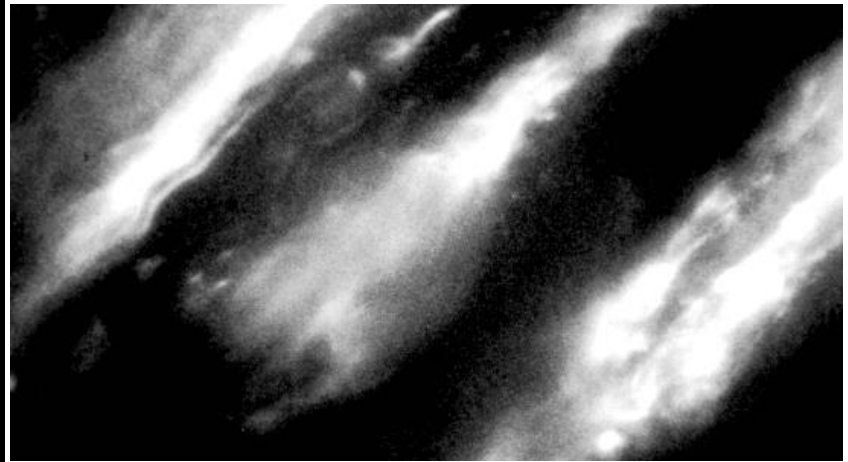
ligament. We hypothesize that this retightening phenomenon does not occur in a clinically lax ACL because such knees undergo recurrent anterior displacements (as opposed to this rat model) and thus the lax human ACL still "sees" intermittent loading.

Our transmission electron microscopy studies of ligamentous tissue showed marked interdigitation of fibroblast cytoplasm with the surrounding collagenous matrix (Figure 6) which would imply that the cells are adequately situated to tighten the surrounding matrix through the actions of their cytoskeleton (Dahners and Banes et al, 1986).

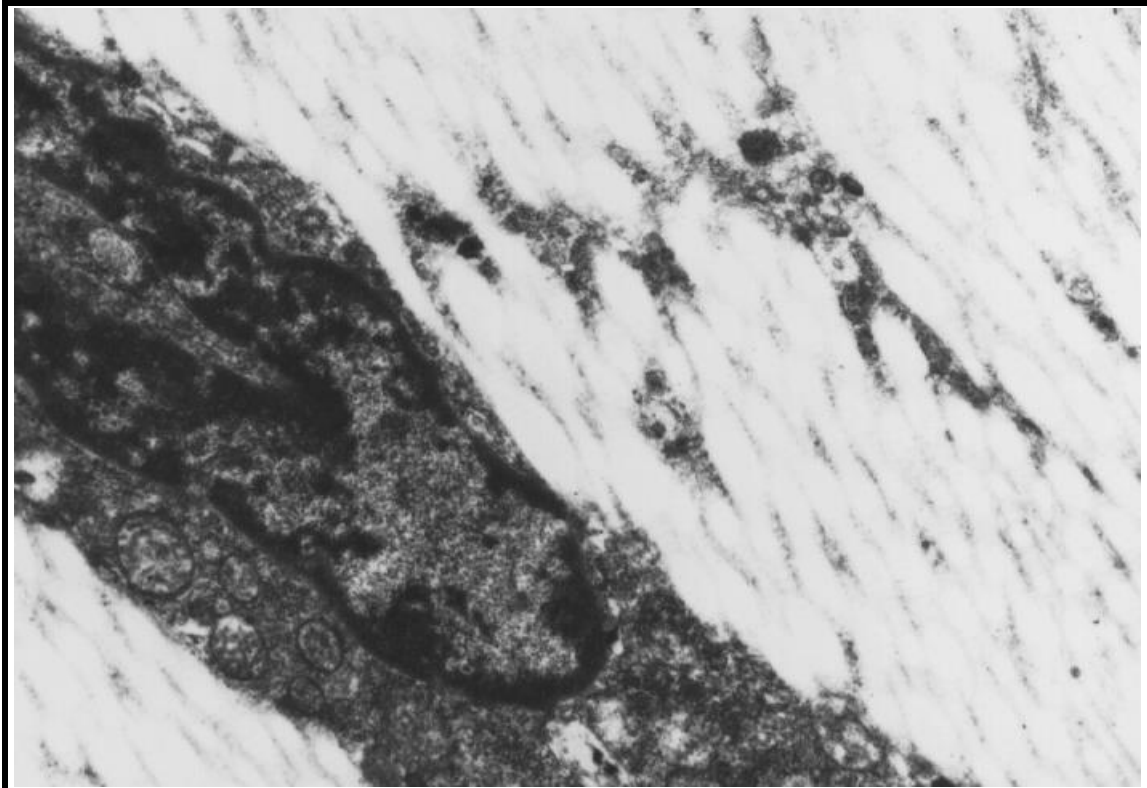
We undertook an experiment to determine whether an absence of the stress generated electrical potentials (SGEPs) produced by ligament loading was a signal for the cells to begin the contracture process. In this experiment, rat knees were immobilized in 150° of flexion and electrodes implanted to deliver simulated SGEPs to the experimental knees (Tart and Dahners, 1989). The simulated SGEPs diminished the contracture process, as compared to controls, but were not able to completely prevent the development of contracture. It is possible that our "simulated" SGEPs may not have been sufficiently similar to "natural" SGEPs, or that some other (non-SGEP) mechanisms are also important. Edwards et. al. have demonstrated that fibroblasts are actually physically deformed by mechanical loading of the ligament (Edwards and Miniaci et al, 1991). In addition (Kenamond and Weinhold et al, 1997 and Shirakura and Ciarelli et al, 1995) calcium flux has been demonstrated in response to mechanical perturbations. Thus fibroblasts may not be solely dependent on intermediary signals (such as SGEPs) to recognize alterations in their mechanical environment.



**Figure 5a: NBD Phalloidin staining of actin in "control" ACL from nonimmobilized left rat knee at three weeks.**



**Figure 5b: NBD Phalloidin staining of actin in contracted ACL from right knee which was immobilized in 150° flexion for three weeks.**



**Figure 6: Transmission electron micrograph of a rabbit MCL fibroblast demonstrating extensive interdigitation of the cytoplasm and the surrounding collagen fibrils.**

Thus, it would appear that the actin cytoskeleton of ligament fibroblasts, coupled with their ability to recognize changes in their mechanical environment, provides a mechanism by which the length of a ligament can be adjusted if it becomes lax. We hypothesize that such mechanisms are responsible for correcting the laxity which would result from micro-stretch injuries to ligaments throughout life (and would otherwise make the ligament excessively lax in later years). The fibroblasts recognize that the ligament matrix is excessively lax when it is not stressed and begin the contracture process to retighten their matrix. The absence of stress generated electrical potentials probably has a role in mediating fibroblast recognition of matrix laxity, but is unlikely to be the only transduction signal.

#### MECHANISMS FOR ALTERATIONS IN LENGTH

Through the work presented above we have established that: 1. ligamentous tissue does change in length during growth and contracture and that it does so in a diffuse fashion distributed throughout the length of the ligament. 2. during contracture this appears to be an active rather than a passive process which involves the fibroblast's actin cytoskeleton, said cytoskeleton being capable of exerting mechanical force on it's matrix, and 3. non-mechanical stimuli such as simulated stress generated electrical potentials are capable of modifying the contracture process. We then investigated the mechanism by which these diffuse length changes take place.

The literature provides evidence that, at least in invertebrates and embryonic tissues, collagen fibrils are discontinuous (i.e. a single fibril does not extend as a single large cross linked molecule from femur to tibia within the MCL). There has been little evidence regarding this issue in non embryonic vertebrates due to the great difficulty in removing intact fibrils from ligaments for study (means for isolating fibrils have been worked out in some marine invertebrates). Trotter and Wofsy have, however, developed some evidence through cross sectional studies that vertebrate collagen fibrils, like invertebrate and embryonic fibrils, are discontinuous and taper to points on both ends (Trotter and Wofsy, 1989). Invertebrate and embryonic vertebrate fibrils have been shown to have a distinct molecular arrangement of the collagen so that the amino terminal ends of the molecules are oriented towards the tapered points of the fibril on both ends with a short segment near the center of each fibril where the molecular polarity reverses (Chapman, 1985; Holmes and Lowe et al, 1994; Matsumura, 1974; Thurmond and Trotter, 1994; Trotter and Thurmond et al, 1994). The discontinuous nature of the fibrils

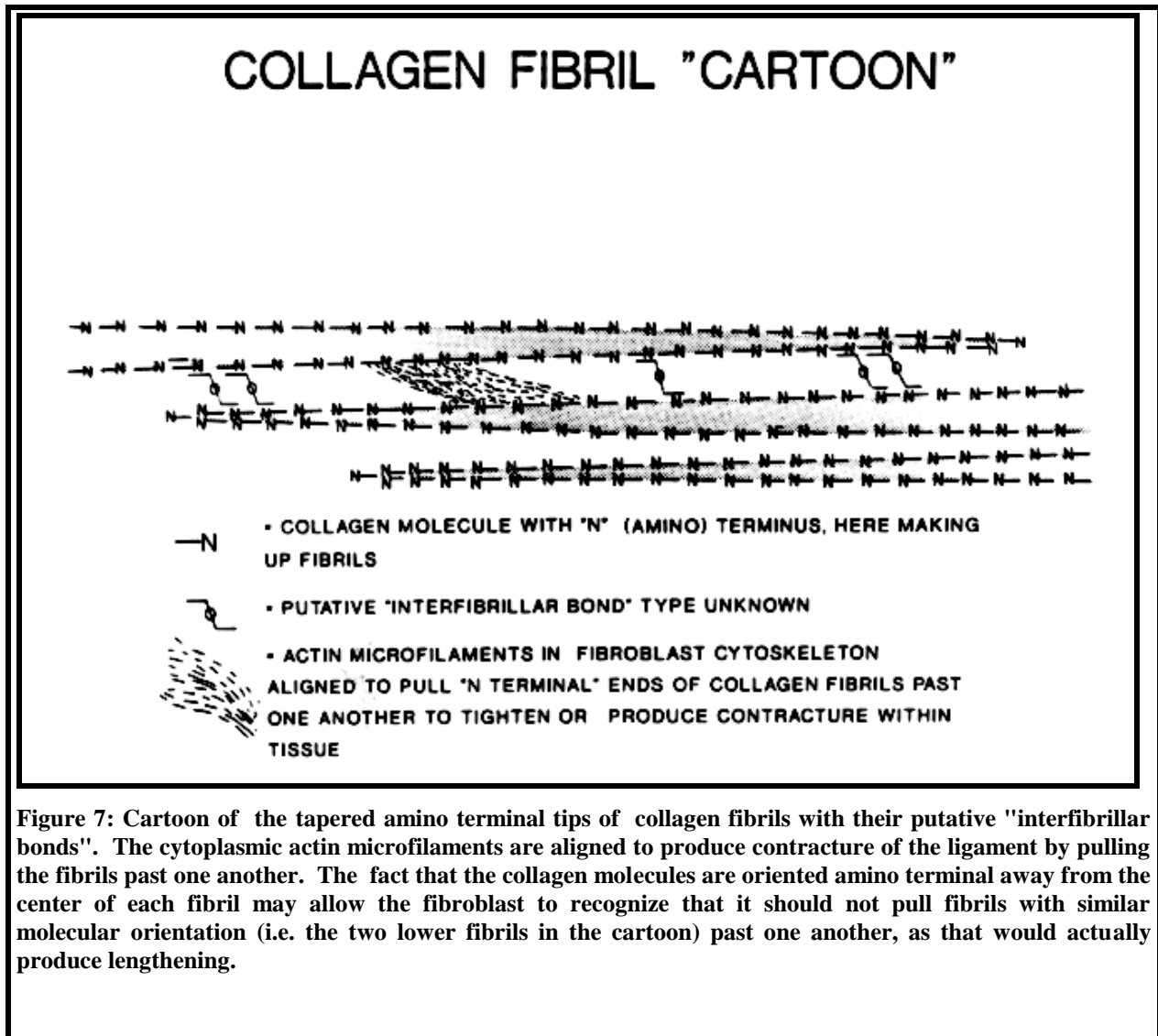
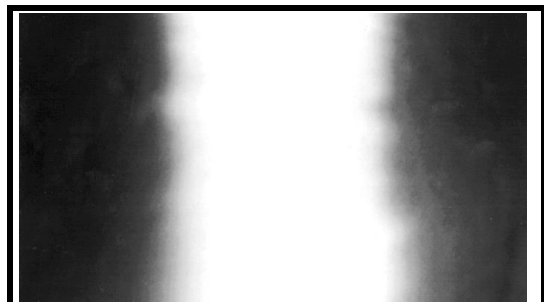


Figure 7: Cartoon of the tapered amino terminal tips of collagen fibrils with their putative "interfibrillar bonds". The cytoplasmic actin microfilaments are aligned to produce contracture of the ligament by pulling the fibrils past one another. The fact that the collagen molecules are oriented amino terminal away from the center of each fibril may allow the fibroblast to recognize that it should not pull fibrils with similar molecular orientation (i.e. the two lower fibrils in the cartoon) past one another, as that would actually produce lengthening.

makes plausible the hypothesis that length changes within dense collagenous tissues occur through the sliding of discontinuous collagen fibrils past one another, producing more overlapping of the fibrils during the process of contracture and less overlapping of the fibrils during the process of lengthening during growth. During growth the length of the fibrils can subsequently be extended and new fibrils added in order to restore sufficient overlap to maintain mechanical integrity. The distinct amino terminal molecular orientation of the fibrils could also serve to orient the fibroblasts so that during the contracture process fibrils would be pulled towards one another producing shortening rather than accidentally "pushed past one another" which could instead produce lengthening (see cartoon, Figure 7) .

For the above hypothesis to be plausible we must further hypothesize readily reversible "interfibrillar bonds" which prevent slipping of fibrils past one another under normal circumstances but can be released by the fibroblasts to allow translation of the fibrils past one another during growth or contracture. While the nature of such putative "interfibrillar bonds" is presently unknown two properties could be presumed: 1) That if vertebrate collagen fibrils have high aspect ratios (length:radius), as do invertebrate fibrils, the strength of the "interfibrillar bonds" would not have to be great because of the extensive overlap of fibrils. 2) Such interfibrillar bonds should be easily released by substances that fibroblasts could secrete into the extracellular milieu.

In order to test the hypothesis that ligament growth and contracture occur through a mechanism of fibril sliding we undertook an experiment in growing and mature Sprague-Dawley rats. Their MCLs were exposed and carefully stained with an extremely thin line of the fluorescent dye dichlorotriazinyl fluorescein (DTAF). Ten rapidly growing (50-75 gm) rats, ten mature controls, and ten mature rats in which a contracture was induced (by distally transecting the MCL) were used in this study. Observation of the DTAF stain line showed a progressive increase in the width of the line in the growth and contracture rats with relative stability in the width of the line in the control group. Fluorescent microscopy showed a "staggered" appearance (Figures 8a and b) to the DTAF mark compatible with fiber sliding in the growth and contracture animals (Woods and Lester et al, 1997).



**Figure 8a: Fluorescent photomicrograph of DTAF line applied transversely on growing rat MCL (at time of application).**



**Figure 8b: Fluorescent photomicrograph of the same DTAF line on growing rat MCL after two weeks (same magnification as 8a above).**

#### “INTERFIBRILLAR BONDING”

We have examined the hypothesis that “interfibrillar bonds” are easily reversible in the following manner. We postulated that a reversal of interfibrillar bonds would cause a substantial weakening of a collagenous tissue and so evaluated the mechanical properties of rat tail tendon after exposure to a variety of agents. In order to evaluate the effects of pH we subjected the

tendons to progressively more acidic solutions. This progressively weakened the tendons as compared to control. This weakening was related to hydrolysis of acid labile crosslinks as stabilizing such crosslinks with  $\text{NaBH}_4$  prevented the weakening of the tendons at lower pH's. However, these affected crosslinks may be intrafibrillar and not necessarily interfibrillar since alteration of either would influence strength. In efforts to alter ionic binding sites we also exposed the tendons to hypo (0x) and hyper (10x) osmolar saline solutions but exposure to these solutions did not weaken tendon. To evaluate the role of calcium we subjected tendons to low concentrations of calcium by exposing them to EDTA and to higher concentrations (2.4 millimolar  $\text{CaHPO}_4$ ) with no decreases in the tendon strength. As Hidaka and Takahashi have shown that neuromodulators affect the "catch" ligament in the sea urchin (a ligament which changes length quite rapidly in this marine invertebrate) we exposed rat tail tendons to the neuromodulators adrenaline chloride and acetylcholine chloride in the same concentrations used in Hidaka and Takahashi's paper without significant weakening of the tendons (Hidaka and Takahashi, 1983). On the hypothesis that decorin or other small proteoglycans may be involved in interfibrillar bonding we digested proteoglycans by exposure to chondroitinase ABC without producing any change in the strength of the tendons. Serendipitously we found that the antibiotic gentamicin (1.0 mg/ml in saline), which we were initially using to prevent bacterial growth during testing, did cause consistent weakening of tendon to  $73 \pm 11\%$  of control strength.

To investigate the gentamicin phenomenon further we determined that gentamicin was a small (15 angstrom) polycation and determined that a four residue polylysine was a polycation of similar size and charge. We then subjected rat tail

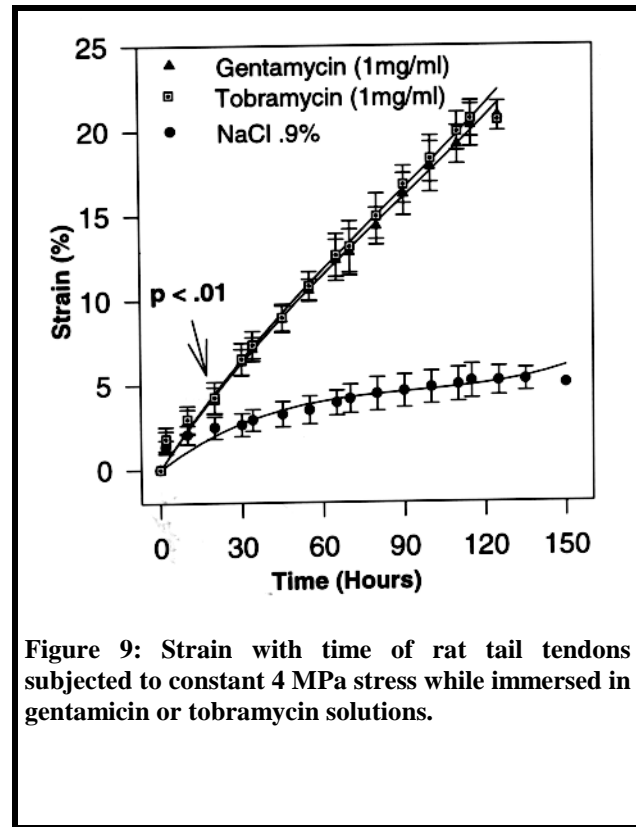


Figure 9: Strain with time of rat tail tendons subjected to constant 4 MPa stress while immersed in gentamicin or tobramycin solutions.

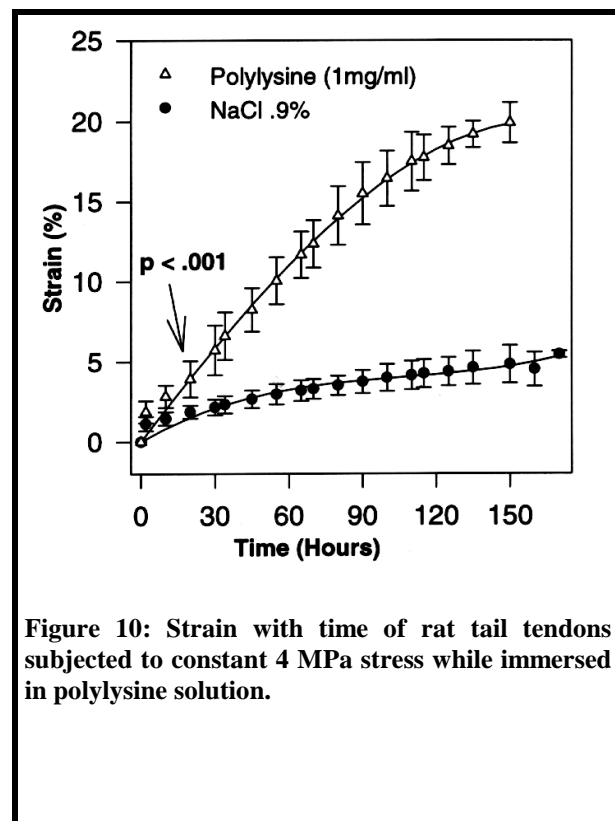


Figure 10: Strain with time of rat tail tendons subjected to constant 4 MPa stress while immersed in polylysine solution.

tendons to constant stress while immersed in control solutions or solutions containing gentamicin, tobramycin 1mg/ml (tobramycin is also a 15 angstrom polycationic antibiotic) or polylysine 1mg/ml. Under a mean stress of 4.0 MPa (range 3.7 to 4.2 MPa) we measured strains greater than 18% (tendons usually rupture before reaching 10% strain) in tendons exposed to the small polycations whereas controls consistently demonstrated strains less than 6% ( $P < 0.001$  at 30 hours, please see Figures 9 and 10). One set of tendons was subjected to 4 MPa stress in gentamicin for 70 hours (achieving 13% strain) and then the gentamicin was removed. Upon removal of the gentamicin the strain rate normalized (slope similar to control) over the next 70 hours, indicating that the increased strain rate in response to the small polycations was reversible (Figure 11).

Based on the above observations we hypothesized that gentamicin weakens interfibrillar bonds. We then attempted to isolate intact vertebrate collagen fibrils following gentamicin treatment. The procedure did not work in uninjured ligaments but specimens harvested at 48 or 96 hours after transection of the MCL of Sprague-Dawley rats did yield some intact collagen fibrils. The harvested injury site specimens were placed in gentamicin or, as a control, in phosphate buffered saline for 72 hours, then vortexed for 1 hour, incubated for an additional 24 hours and vortexed again for 1 hour. Fluid from the specimens was examined with transmission electron microscopy. While the phosphate buffered saline specimens yielded only broken fibrils, the gentamicin exposed specimens yielded both intact (Figure 12a) and broken fibrils that were structurally consistent with those identified in echinoderms and in chick embryo tendons in that they tapered to points on both ends and were molecularly bipolar: i.e. their collagen molecules were oriented with their amino termini towards

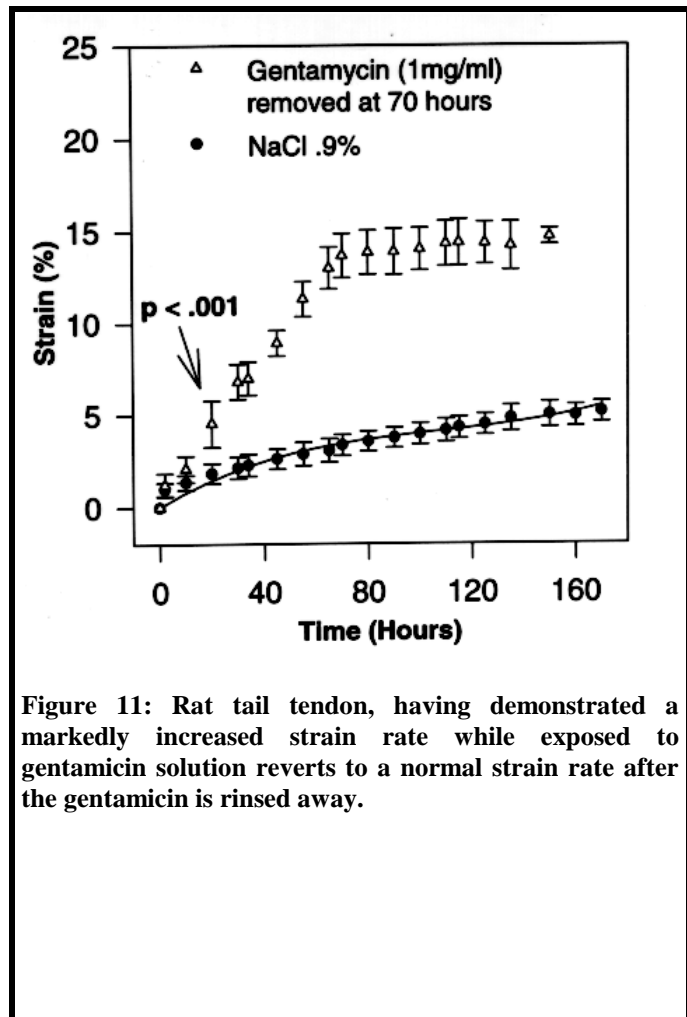


Figure 11: Rat tail tendon, having demonstrated a markedly increased strain rate while exposed to gentamicin solution reverts to a normal strain rate after the gentamicin is rinsed away.

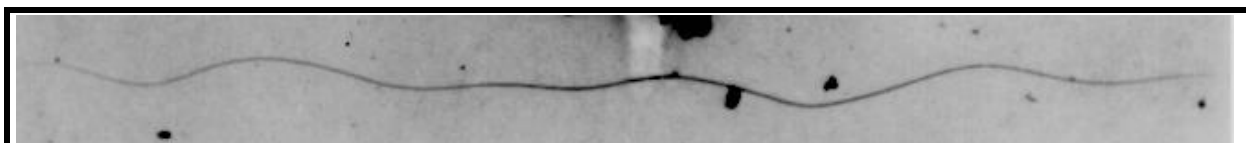
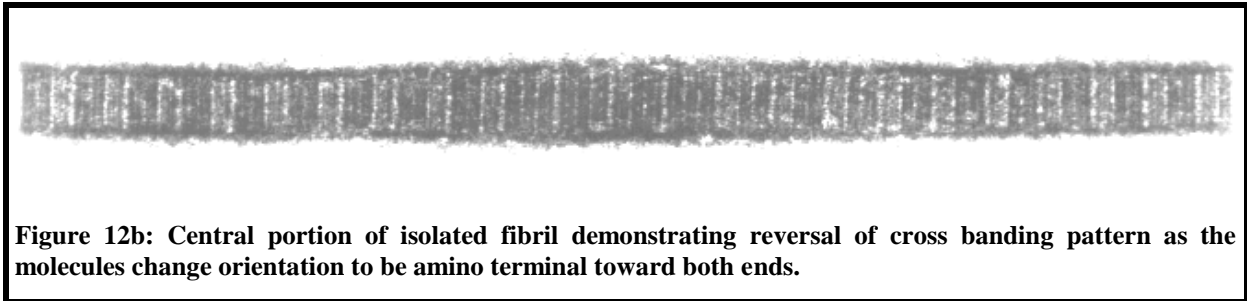


Figure 12a: Collagen fibril isolated from healing rat MCL. Note that it tapers to points on both ends.



nearest fibril end with a zone in the center where the molecules changed orientation (Figure 12b) as determined by examination of the cross banding pattern (Chapman, 1985). Although only four completely intact fibrils were identified, no method has previously succeeded in isolating intact fibrils from a post fetal vertebrate tissue, thus the presence of intact and mostly intact fibrils supports the hypothesis that treatment with gentamicin results in the release of interfibrillar bonds in some manner (DeVente and Lester et al, 1997).



**Figure 12b: Central portion of isolated fibril demonstrating reversal of cross banding pattern as the molecules change orientation to be amino terminal toward both ends.**

#### SUMMARY OF CLINICAL RELEVANCE

We believe that an understanding of the mechanisms of length changes in dense collagenous tissues is critically important to musculoskeletal research and to orthopaedic surgery. Excessive growth of ligaments may be an etiology for joint laxity and ligament contracture is certainly a common cause for musculoskeletal dysfunction. The work presented here documents that both growth and contracture are phenomena occurring diffusely throughout the structure of ligaments rather than at an isolated “growth plate”. Mechanical stress seems to play an important role in modulating growth as well as the absence of stress having major importance in the development of contracture. The lack of mechanical stress leading to contracture may be recognized in part by the cells through an absence of stress generated electrical potentials. There is a significant amount of evidence supporting the hypothesis that changes in length occur through the sliding of fibrils past one another. During contracture the contractile actin cytoskeleton of the fibroblasts is active and presumably provides the motive force in sliding the fibrils past one another while the ligament is shortening. Although the nature of the “interfibrillar bonds” which bind one fibril to another to prevent such sliding during normal circumstances is unknown as yet, it appears that it can be modulated by small polycations. Identification of more effective mechanisms for modulating interfibrillar bonding may allow the development of mechanisms by which ligament length can be purposefully modified, allowing alternatives to current therapies for ligament laxity and contracture.

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#### CONFLICTS OF INTEREST

None.

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SUMMARY OF INVESTIGATOR'S WORK LEADING TO THIS DOCUMENT

The primary investigator began work on this topic while he was still an Orthopaedic resident in 1982. Dr. Lester joined him in these studies in 1985. Numerous students, residents, and collaborators have helped with various portions of this work over the years since then. We would be pleased to list them all as authors but are following the committee's recommendation that only those responsible for "significant ongoing contributions" be listed. Published work is referenced below.

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## FIGURE LEGENDS

Figure 1: Final length of the studied intermarker intervals as a mean percentage of the original length of the intervals ( $\pm$  one standard deviation). All intervals have lengthened, the distal intervals more than the proximal ones.

Figure 2: Four outcomes hypothesized for the growth response of the MCL to arrest of underlying physis as demonstrated in a marking suture study.

Figure 3: Rat fibroblast stained with NBD Phalloidin to demonstrate its prominent actin cytoskeleton.

Figure 4: Rat fibroblasts grown on a thin silastic membrane. Note that they are producing wrinkles in the underlying silastic.

Figure 5a: NBD Phalloidin staining of actin in "control" ACL from nonimmobilized left rat knee at three weeks. 5b: NBD Phalloidin staining of actin in contracted ACL from right knee which was immobilized in 150° flexion for three weeks.

Figure 6: Transmission electron micrograph of a rabbit MCL fibroblast demonstrating extensive interdigitation of the cytoplasm and the surrounding collagen fibrils.

Figure 7: Cartoon of the tapered amino terminal tips of collagen fibrils with their putative "interfibrillar bonds". The cytoplasmic actin microfilaments are aligned to produce contracture of the ligament by pulling the fibrils past one another. The fact that the collagen molecules are oriented amino terminal away from the center of each fibril may allow the fibroblast to recognize that it should not pull fibrils with similar molecular orientation (i.e. the two lower fibrils in the cartoon) past one another, as that would actually produce lengthening.

Figure 8a: Fluorescent photomicrograph of DTAF line applied transversely on growing rat MCL (at time of application). 8b: Fluorescent photomicrograph of the same DTAF line on growing rat MCL after two weeks (same magnification as 8a above).

Figure 9. Strain with time of rat tail tendons subjected to constant 4 MPa stress while immersed in gentamicin or tobramycin solutions.

Figure 10. Strain with time of rat tail tendons subjected to constant 4 MPa stress while immersed in polylysine solution.

Figure 11. Rat tail tendon, having demonstrated a markedly increased strain rate while exposed to gentamicin solution reverts to a normal strain rate after the gentamicin is rinsed away.

Figure 12a: Collagen fibril isolated from healing rat MCL. Note that it tapers to points on both ends. 12b: Central portion of isolated fibril demonstrating reversal of cross banding pattern as the molecules change orientation to be amino terminal toward both ends.

**TABLES**

**Table 1**  
Percent of Growth of Four  
Longitudinal Ligament Intervals in  
Seven Rabbit Deltoid Ligaments

Interval	% Growth
Proximal	37 ± 9
Midproximal	36 ± 5
Middistal	34 ± 11
Distal	33 ± 19

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Values are given as means ± SE.

**Table 2**  
Ligament Elongation in Growing Rabbits  
With and Without Tension

	Control (Fibular Head Still Attached)	Sham (Rubber Band Lax)	Experimental (Rubber Band Under Tension)
6-week old rabbits	12 ± 8%	16 ± 4%	NA
6-week old rabbits	14 ± 6%	NA	29 ± 11%
4-week old rabbits	79 ± 5%	NA	140 ± 18%

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Values are given as means ± SE.

**Table 3**  
Ligament Elongation in Adult Rabbits  
With and Without Tension and/or Growth Hormone

	Control (Fibular Head Still Attached)	Sham (Rubber Band Lax)	Experimental (Rubber Band Under Tension)
Adults with GH	2 ± 4%	-2 ± 5%	3 ± 7%
Adults without GH	NA	4 ± 6%	5 ± 9%

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Values are given as means ± SE.