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Collagen self-assembly and the development of tendon mechanical properties

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Abstract

The development of the musculoskeleton and the ability to locomote requires controlled cell division as well as spatial control over deposition of extracellular matrix. Self-assembly of procollagen and its final processing into collagen fibrils occurs extracellularly. The formation of crosslinked collagen fibers results in the conversion of weak liquid-like embryonic tissues to tough elastic solids that can store energy and do work.

Collagen fibers in the form of fascicles are the major structural units found in tendon. The purpose of this paper is to review the literature on collagen self-assembly and tendon development and to relate this information to the development of elastic energy storage in non-mineralizing and mineralizing tendons. Of particular interest is the mechanism by which energy is stored in tendons during locomotion.

In vivo, collagen self-assembly occurs by the deposition of thin fibrils in recesses within the cell membrane. These thin fibrils later grow in length and width by lateral fusion of intermediates. In vitro, collagen self-assembly occurs by both linear and lateral growth steps with parallel events seen in vivo; however, in the absence of cellular control and enzymatic cleavage of the propeptides, the growth mechanism is altered, and the fibrils are irregular in cross section.

Results of mechanical studies suggest that prior to locomotion the mechanical response of tendon to loading is dominated by the viscous sliding of collagen fibrils. In contrast, after birth when locomotion begins, the mechanical response is dominated by elastic stretching of crosslinked collagen molecules.

1. Introduction

Elastic energy storage in tendons in the legs and feet of many animals is an important mechanism that saves substantial quantities of muscular energy during locomotion (Alexander, 1983, 1984). During normal gait, potential energy is stored as strain energy in the muscles and tendons that are stretched by impact with the ground (Alexander, 1983, 1984). Elastic recoil, primarily by the tendons, converts most of the stored energy back to kinetic energy. Elastic energy storage in tendons has been studied in several animal models. In the pig, the digital flexor tendons are involved in the elastic storage of strain energy (Shadwick, 1990); the amount of elastic energy stored in the digital flexor tendons decreases with age after the animal reaches maturity. In the turkey, direct measurement of force and fiber length in the lateral gastrocnemius muscle reveals that the active muscle produces high force but little work while the tendon produces much of the work because of elastic deformation and recovery (Roberts et al., 1997).

The mechanism by which elastic energy is stored in tendon during locomotion is not well understood. It has been shown that both the axial rise per amino acid residue and the *D* period increase with increased tendon mechanical deformation suggesting that molecular stretching and slippage occur during deformation (Mosler et al., 1985; Sasakai and Odajima, 1996a,b). The purpose of this review is to attempt to describe the molecular and supramolecular steps associated with collagen self-assembly and tendon development, and to analyze how these steps relate to the ability of tendons to store energy elastically.

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2. Molecular structure of collagens

The mechanical properties of tendon are a direct consequence of the constituent components and how these components are arranged. Tendon is primarily made up of cells, collagen fibers, proteoglycans and water. Collagen is the most abundant protein in tendon and forms the essential mechanical building blocks in the musculoskeletal system. It can be found in both fibril and non-fibril forming forms. The fibril-forming collagens provide the structural framework of tissues; they include types I, II, III, V, and XI collagens (see Silver and Christiansen, 1999). All of the fibril forming collagens self-assemble into cross striated fibrils with a characteristic 67 nm repeat; they all share a triple helical region that is roughly 1000 amino acid residues long with a length of about 300 nm (see Silver and Christiansen, 1999). These collagens are synthesized within cells in a precursor form termed procollagen which has amino and carboxyl terminal non-helical ends that are about 15.0 and 10.0 nm long, respectively (Fig. 1). In addition, fibril-associated collagens with interrupted triple helical sequences (FACIT collagens) such as type XII collagen are found on the surface of collagen fibrils and may connect fibrillar collagens to other components of the extracellular matrix (Keene et al., 1991).

Tendon is composed predominantly of type I collagen, although it contains small amounts of types III and V (Silver et al., 1992). It has been reported that the type I collagen molecule is rod-like with little flexibility and high mechanical strength (Engle, 1997). The rod-like behavior of type I collagen was first established based on measurement of the translational diffusion coefficient (Fletcher, 1976; Thomas and Fletcher, 1979; Silver et al., 1979); however, later measurements indicated that the type I collagen molecule had numerous bends and was not completely rigid (Silver and Birk, 1984).

The molecular basis for the flexibility of type I collagen comes from sequences that lack the amino acids proline and hydroxyproline (Fig. 1). These sequences are the sites where bends can occur in the triple helix. Five sites have been identified by Hofmann et al. (1984) based on electron microscopic images; these sites occur at characteristic distances of 30–45, 90–105, 150–157.5, 210–217.5 and 270–277.5 nm from the C terminus of the molecule. Results of a recent modeling study suggest that sequences without proline and hydroxyproline are able to form internal loops (Paterlini et al., 1995) that give these regions more flexibility than the other regions of the triple helix. Stereochemical maps constructed for dipeptides containing amino and imino acid residues also suggest that in the absence of proline and hydroxyproline, the number of available conformations and consequently the flexibility are increased (Silver, 1987; Silver and Christiansen, 1999). A flexibility profile as a function of axial displacement for the type I collagen triple-helix is shown in Fig. 1. This diagram suggests that the collagen triple-helix can be considered a composite of regions with varying degrees of stiffness; regions of the molecule devoid of proline and hydroxyproline appear to have the highest flexibility whereas regions with the sequence Gly–Pro–Hyp are very rigid. This variation in molecular flexibility affects collagen self-assembly as well as the resulting mechanical properties of tendons. Results of recent modeling studies suggest that fibril diameters appear to be inversely related to collagen molecular flexibility (Silver et al., 2001c).

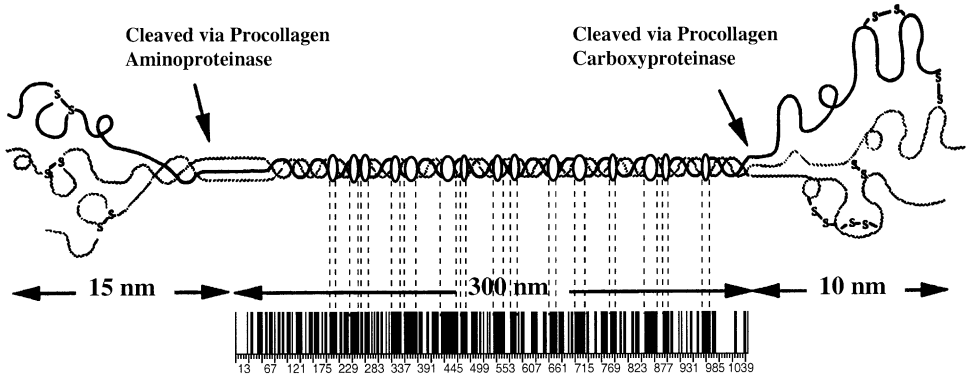

Fig. 1. Diagram of procollagen type I molecule. The procollagen molecule is shown at the top and consists of aminopropeptides (left-hand portion of molecule at top), an amino non-helical end (straight portion), a triple helical region, a carboxylic non-helical end and a carboxylic propeptide (right-hand side end of molecule). The amino (N-) and carboxylic (C-) propeptides are cleaved by specific proteinases during collagen self-assembly. The circles in the triple helix represent major sequences devoid of proline and hydroxyproline that are the likely sites of folds where flexibility is introduced into a normally rigid helix. The striated pattern shown below the helical portion of the molecule is a diagrammatic representation of the flexible (dark bands) and rigid regions (light bands) found in the triple helix. Note the ends of the triple helix are rigid while sequences towards the center of the molecule are more flexible.

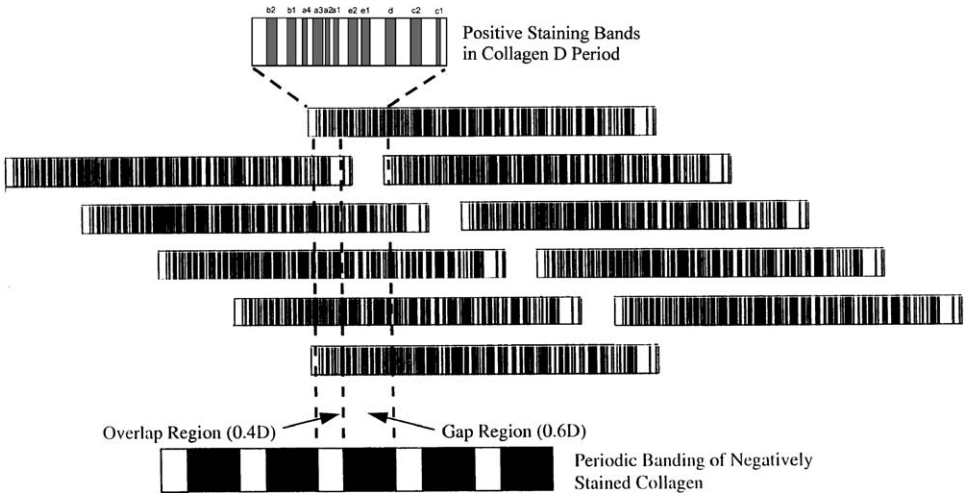

Fig. 2. Diagram of quarter stagger packing pattern of type I collagen molecules in tendon. Neighboring collagen molecules, represented by a series of light and dark bands as shown in Fig. 1, are shown staggered with respect to their nearest neighbors. In tendon, collagen molecules are staggered by a distance D after they are self-assembled into fibrils in tissues. When collagen is stained negatively and then viewed in the electron microscope, a series of light and dark bands are observed across the axis of the fibril which result from the stain penetrating only the hole region and not the molecule (light–dark repeat shown at bottom of figure). The distance D in tendon is 67 nm and is made up of a hole region of about $0.6D$ and an overlap region of about $0.4D$. The D period is the characteristic fingerprint of fibrous collagen. When collagen is stained with heavy metals and viewed in the electron microscope the D period is observed from either the light–dark repeat (negative staining pattern) seen or by the repeat of 12 bands (bands b2 through c1 at top of figure) by positive staining. In positive staining, the stain penetrates into the molecule. The positive staining pattern arises when the stain binds directly to the charged amino acid residues on the molecule. Note proteoglycans are observed bound to the d and e bands in the positive staining pattern.

Small amounts of types III and V collagens are found in mixtures with type I in a single fibril suggesting that tissue specific differences in mechanical properties reflect different mixtures of these collagen types (Birk et al., 1991). It has recently been postulated that the type III collagen molecule is more flexible than the type I collagen molecule also suggesting that mixing collagen types would alter the elastic modulus of collagen fibrils (Silver et al., 2002a). Immunolocalization and/or chemical crosslinking studies have shown that collagen types I and V can be present as "heteropolymers" (Fitch et al., 1984, 1988; Birk et al., 1988). Although the type V molecule is somewhat larger than that of type I, due to the N-terminal domain, type V molecules are assembled into typical "quarter-staggered" fibrils characteristic of type I collagen (Fig. 2). The formation of mixtures of such "heterotrophic" or "heterotypic" fibrils provides a means to modulate self-assembly and modify mechanical properties (Birk et al., 1991). The presence of a larger N-terminal domain is likely to modify fibrillar packing and slippage of collagen molecules during mechanical deformation. Experimental observations on a mouse model with a structurally abnormal $\alpha 2$(V) collagen chain indicate that the resultant collagen fibrils are loosely packed in skin. This is consistent with the hypothesis that the large N-terminus of the type V collagen molecule may regulate growth of skin fibrils (Andrikopoulos et al., 1995).

3. Collagen assembly in developing tendon

The ability of collagen molecules to assemble into crosslinked fibrils is an important requirement for the development of tissue strength. Although the process is under cellular control, the tendency for collagen molecules to form crossstriated fibrils is a property of the molecular sequence, as discussed in more detail in the section on self-assembly.

Tendon is a multi-unit hierarchical structure that contains collagen molecules, fibrils, fibril bundles, fascicles and tendon units that run parallel to the geometrical axis (Elliott, 1965) as diagrammed in Fig. 3. The fundamental structural element in tendon is type I collagen in the form of fibrils. Collagen is synthesized in the precursor form, procollagen, which contains non-triple helical extensions at both ends (Fessler and Fessler, 1978; Prockop et al., 1979) (Fig. 1). The presence of amino- (N) and carboxylic terminal (C) extensions on the collagen molecule have been shown to limit self-assembly of procollagen to about 5 molecules (Berg et al., 1986). Removal of the N- and C-propeptides by specific proteinases occurs prior to final fibril assembly (Tuderman et al., 1977). The C-propeptides are essential for both the initiation of procollagen molecular assembly from the constituent chains (Engel and Prockop, 1991) and lateral assembly of procollagen molecules. Procollagen molecular assembly in vivo

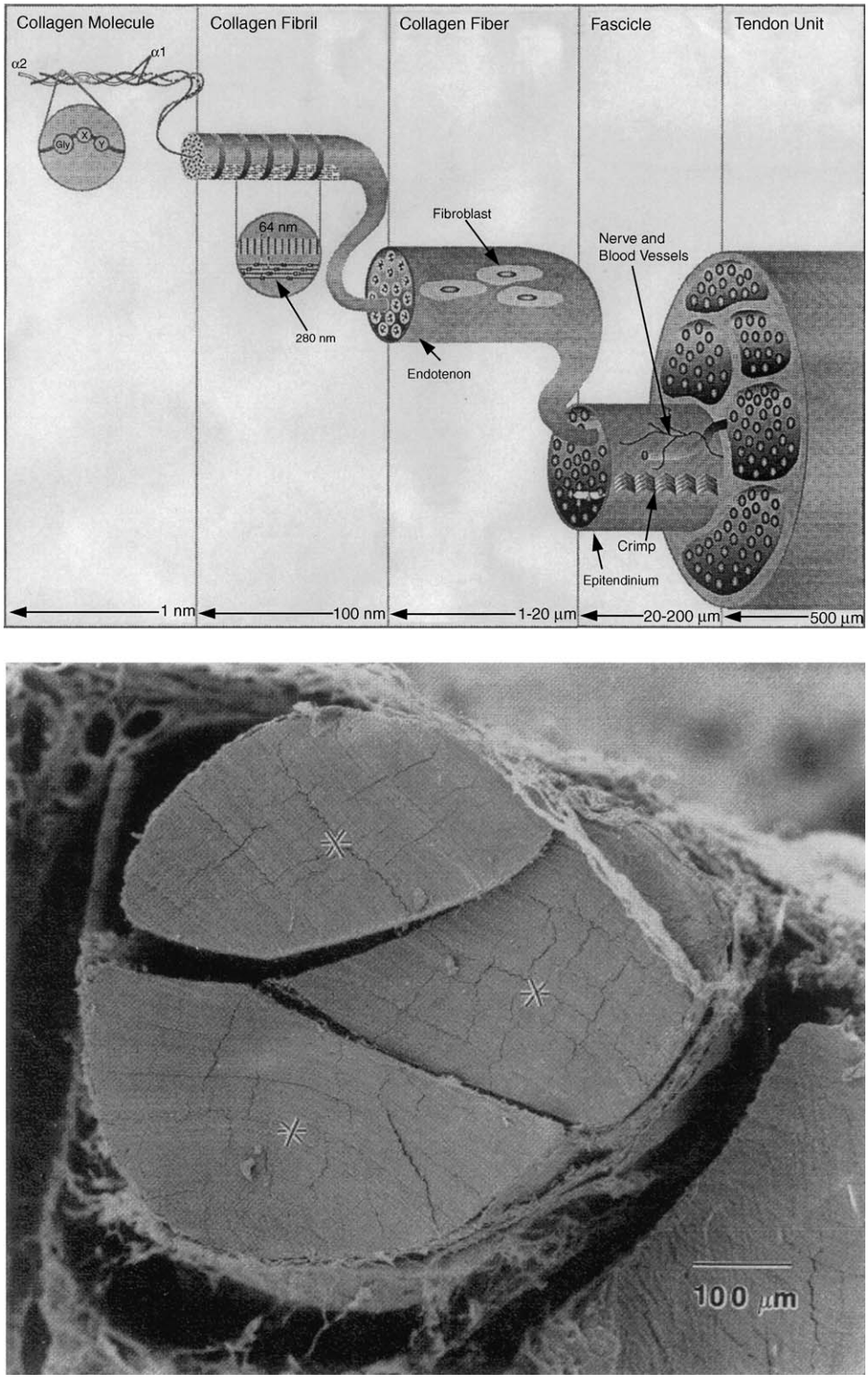

Fig. 3. Structural hierarchy in tendon. (Top) Diagram illustrating the relationship between collagen molecules, fibrils, fibers, fascicles and tendon units. Although the diagram does not show fibril subunits, collagen fibrils appear to be self-assembled from intermediates that may be integrated within the fibril. (Bottom) Scanning electron micrograph of rat tail tendon showing fascicle units (asterisk) that make up the tendon.

initiates within intracellular vesicles (Trelstad and Hayashi, 1979). These vesicles are thought to move from regions within the Golgi apparatus to deep cytoplasmic recesses where they discharge their contents. Results of studies on embryonic skin suggest that the N-propeptides remain attached to fibrils 20–30 nm in

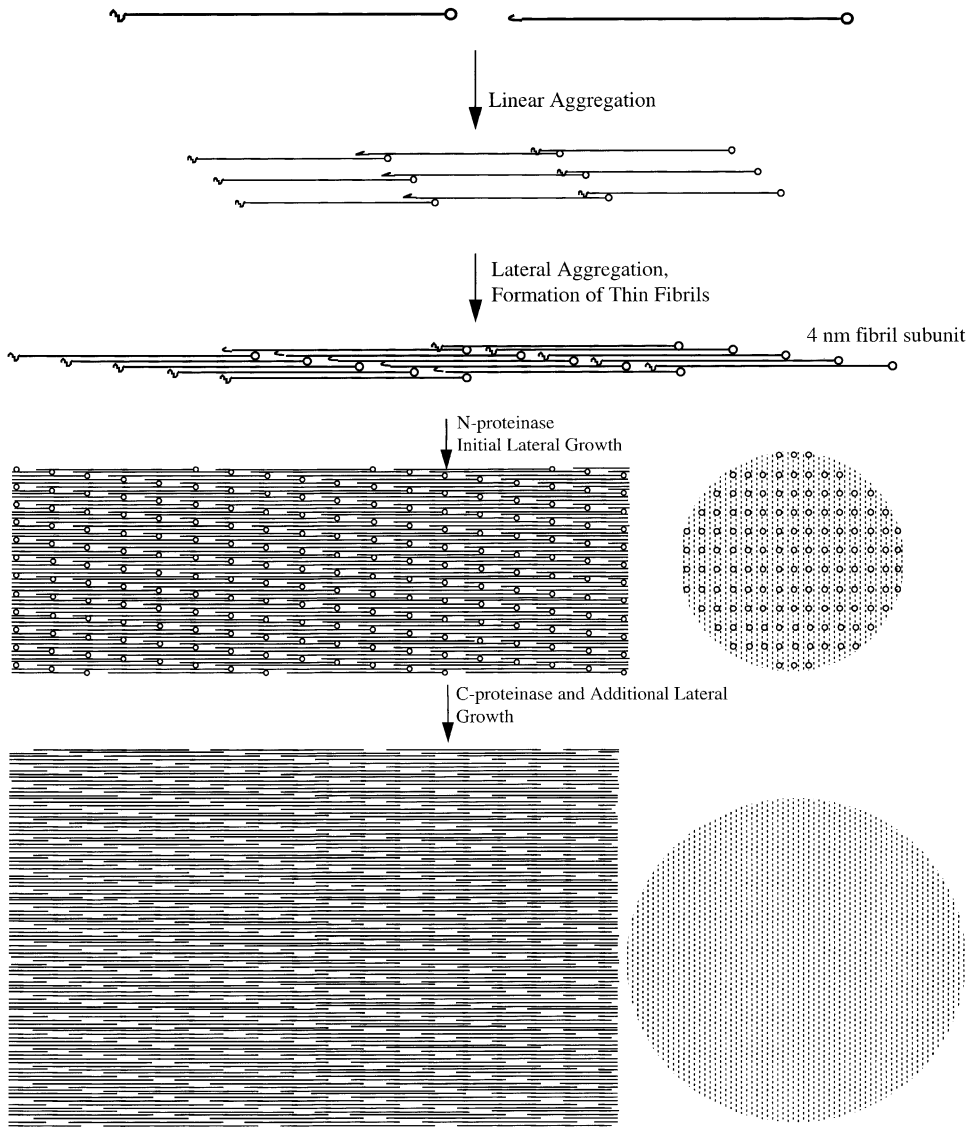

Fig. 4. Diagram showing role of N- and C-propeptides in collagen self-assembly. The procollagen molecule is represented by a straight line with bent (N-propeptide) and circular (C-propeptide) regions. Initial linear and lateral aggregation is promoted by the presence of both the N- and C-propeptides. In the presence of both propeptides lateral assembly is limited and the fibrils are narrow. Removal of the N-propeptide results in lateral assembly of narrow fibrils; removal of the C-propeptide results in additional lateral growth of fibrils. As indicated in the diagram, the presence of the N- and C-propeptides physically interferes with fibril formation.

diameter after collagen is assembled; however, after the N-propeptide is cleaved, fibril diameters appear to increase suggesting that the N-propeptide is associated with initiation of fibrillogenesis. The C-propeptide is removed before further lateral fibril growth occurs (Fleishmajer et al., 1983) (Fig. 4).

Fibrillogenesis of type I collagen is specifically impaired in the skin of animals with a disease termed dermatosparaxis (Lenaers et al., 1971). In this disease the N-propeptide of the pro α1(I) chain is not cleaved resulting in skin that is easily torn. Studies on dermatosparactic calf skin, in which 57% of the collagen molecules have intact N-propeptides, suggest that the presence of the N-propeptide on the fibril surfaces prevents tight packing of the collagen fibrils and results in skin fragility (Watson et al., 1998). Mass mapping of the dermatosparatic collagen fibrils shows that the N-propeptides are in a bent-back conformation that is within the overlap region (Watson et al., 1998). The finding of intact N-propeptides on fibril surfaces is also observed in cell cultures of skin fibroblasts from a patient with Ehlers Danlos syndrome type VII (Watson et al., 1992). Partial cleavage of the N-propeptide allows the N-propeptide to become incorporated within the body of the fibrils (Watson et al., 1992). This finding led these authors to propose that the type 1 procollagen N-propeptides facilitate the fusion of small diameter fibrils (Watson et al., 1998).

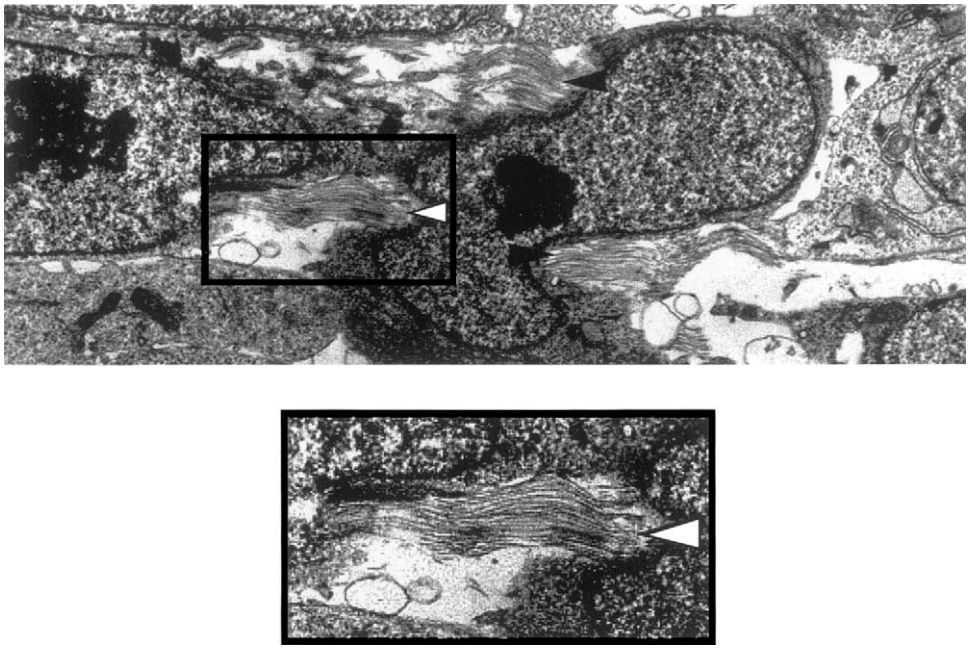

Fig. 5. Directed cellular self-assembly of axial collagen fibrils during chick tendon development. Transmission electron micrograph showing collagen fibrils (see arrow in box) from a 7-day-old chick leg extensor tendon that appear to be connecting two fibroblasts during tendon development. Inset shows a high magnification view of the collagen fibrils that originate from invaginations in the cell membranes on either side of the fibril. The collagen fibrils shown are about 50 mm in diameter. The micrograph was adapted from McBride (1984).

The C-propeptide of fibril-forming collagens appears to regulate later steps in the assembly of procollagen into fibrils; it is removed from small diameter fibrils during growth (Ruggerio et al., 1988) possibly during fibril fusion. The C-propeptide has been observed in fibrils with diameters between 30 and 100 nm (Fleischmajer et al., 1987) suggesting that it is involved in initiation and lateral growth of fibrils (Fig. 4). Procollagen and the intermediates pN-collagen (containing the N-propeptide) and pC-collagen (containing the C-propeptide) are present in developing tendon up to 18 days embryonic (Fleischmajer et al., 1988). Collagen oligomers isolated from developing chick tendons include 4-D staggered dimers of collagen molecules suggesting that this is a preferred molecular interaction for initiation of collagen fibrillogenesis in vivo (Fleischmajer et al., 1991). About 50% of the fibrils formed in 18-day-old chick embryos are bipolar (molecules run in both directions along the axis of the tendon) while the other half are unipolar. Analysis of the staining pattern of fibrils reveals the axial zone of molecular polarity to be highly localized (Holmes et al., 1994).

During chick tendon development the structure and mechanical properties of tendon change rapidly (Torp et al., 1975; McBride, 1984; McBride et al., 1985, 1988). The morphology of embryonic development of collagen fibrils in chick tendon has been studied and characterized extensively (Elliott, 1965; McBride et al., 1985, 1988; Birk et al., 1989a,b, 1995, 1997). Two levels of structural organization of the collagen fibrils seem to occur during development of chick hind limb extensor tendons (McBride et al., 1985). Along the axis of the tendon, cytoplasmic processes of one or more axial tendon fibroblasts are observed to direct formation of groups of short collagen fibrils that appear to connect cells together (Fig. 5). Groups of axial tendon cells are encircled by a second type of fibroblast that forms bundles of collagen fibers. This type of cell encircles groups of collagen fibrils with a sheath that separates fascicles. Initially, axial tendon cells appear at both ends of growing fibrils (Fig. 5). Once the fibrils begin to elongate they are then packed closely side-to-side (Fig. 6). Later a planar crimp is introduced into collagen fibrils perhaps by the contraction of cells at the ends of the fibrils or by shear stresses introduced by tendon cells between layers of collagen fibrils (Fig. 7). Results of recent modeling studies suggest that the molecule and fibril have many points of flexibility (Silver et al., 2001c) where crimp could develop.

In cross section, collagen fibers are made up of individual fibrils that appear to be released from invaginations in the cell membrane (Fig. 8). Additional collagen diameter growth appears to occur by addition of materials that appear to originate inside the Golgi apparatus. Later during lateral growth, these invaginations in the cell membrane disappear causing lateral fusion of fibrils (Fig. 9). Macroscopically this results in increases in fibril diameter and length (Fig. 10).

Birk and coworkers have studied the manner in which collagen fibrils are assembled from fibril "segments" in

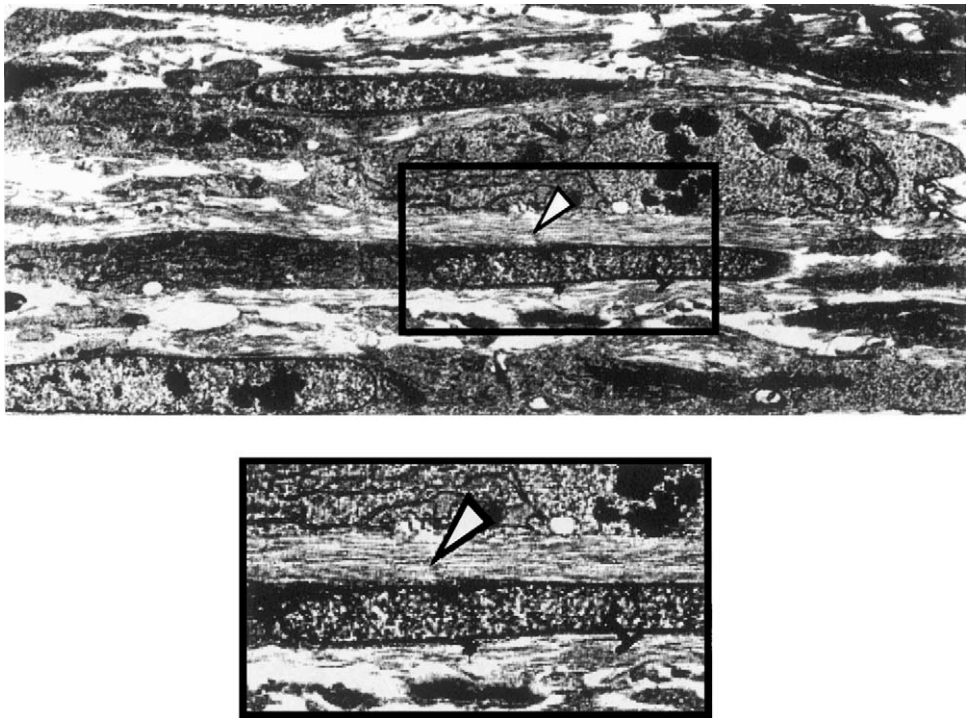

Fig. 6. Lateral condensation of axial collagen-fibrils and alignment of tendon fibroblasts. Transmission electron micrograph showing collagen fibrils from a 10-day-old chick leg extensor tendon. Note the fibrils (see arrow) and fibroblasts appear to be more highly aligned and densely packed compared to the same structures at day 7. Fibrils shown have diameters of about 50 nm. Insert shows a high magnification view of the relationship between the collagen fibrils and the cell surfaces on either side. This micrograph was adapted from McBride (1984).

developing chick tendon (Birk et al., 1997). During development, fibril segments are assembled in extracytoplasmic channels defined by the fibroblast. In 14-day-old chick embryos, tendon fibril segments are deposited as units 10–30 μm in length. These segments can be isolated from tendon and studied by electron microscopy (Birk et al., 1995). Holmes and coworkers have shown that fibrils from 12-day-old chick embryos grow in length at constant diameter (Holmes et al., 1998), and that end-to-end fusion requires the C terminal end of a unipolar fibril (Graham et al., 2000). By 18 days, embryonic fibril growth occurs at both fibril ends and is associated with increased diameter (Holmes et al., 1998). Since fibril segments at 18 days cannot be isolated from developing tendon it is likely that fibril fusion and crosslinking occur simultaneously.

Fibril segments appear to be intermediates in the formation of mature fibrils (Birk et al., 1997) and range in length from 7 to 15 μm in 14-day-old embryonic tendon (Birk et al., 1989a,b). Between 14 and 17 days the bundles begin to branch and undergo rotation over several micrometers (Birk et al., 1989a,b) and the segments increase in length up to 106 μm (Birk et al., 1995). A rapid increase in length and diameter is seen between days 16 and 17 (Birk et al., 1995) and is consistent with the rapid increase in tendon ultimate tensile strength (McBride et al., 1988). Although the fibril packing density of collagen does not change prior to and just after birth, the mean collagen fibril width increases and the cell volume fraction decreases (McBride et al., 1985). This is believed to be associated with growth by fibril fusion (Birk et al., 1997).

In mature tendon, collagen fibril bundles (fibers) have diameters between 1 and 300 μm and fibrils have diameters from 20 to over 280 nm (Silver et al., 1992) (Fig. 3). The presence of a crimp pattern in the collagen fibers has been established for rat tail tendon (Diamant et al., 1972) as well as for patellar tendon and anterior cruciate ligament (Yahia and Drouin, 1969); the specific geometry of the pattern, however, differs from tissue to tissue. It is not clear that the crimp morphology of tendon is actually present in tendons that are under normal resting muscular forces.

4. Role of proteoglycans (PGs) in tendon development

Tendon contains a variety of proteoglycans (PGs) including decorin (Scott, 1993) a small leucine-rich PG that binds specifically to the d band of positively stained type I collagen fibrils (Scott and Orford, 1981) as well as hyaluronan, a high molecular weight polysaccharide. Other small leucine-rich PGs include biglycan, fibromodulin, lumican, epiphycan and keratocan (Iozzo and Murdoch, 1996). In mature tendon the PG(s) are predominantly proteodermochondran sulfates (Scott,

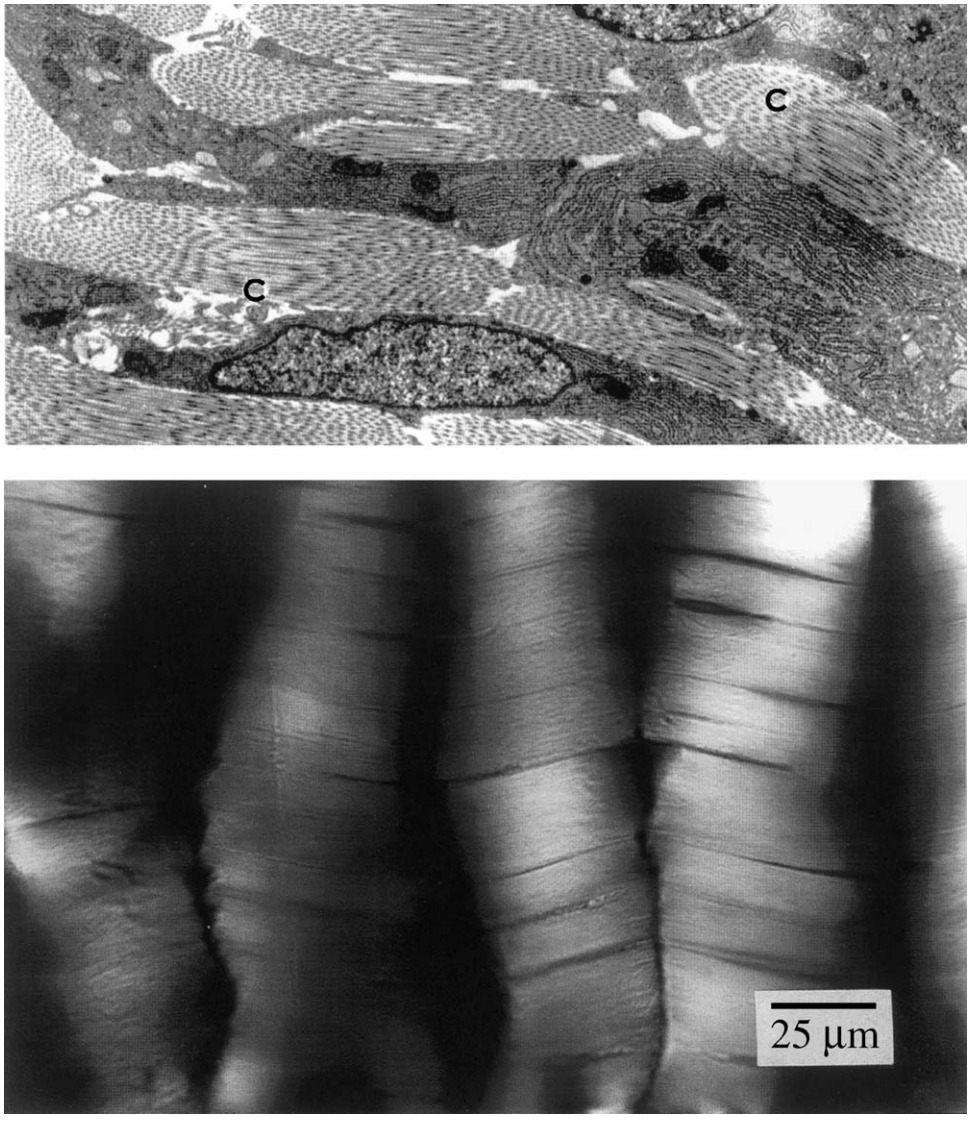

Fig. 7. Formation of crimp in axial collagen fibrils during development of chick extensor tendon. (Top) Transmission electron micrograph showing collagen fibrils (C) from a 17-day-old chick leg extensor tendon. Note the fibrils appear to be going in and out of the plane of section consistent with the formation of a crimped planar zig-zag pattern. Fibrils shown have diameters of about 100 nm. This micrograph was adapted from McBride (1984). (Bottom) Polarized light micrograph illustrating the crimp pattern seen in a rabbit Achilles tendon.

1993). PGs are seen as filaments regularly attached to collagen fibrils in electron micrographs of tendon stained with Cupromeronic blue (Cribb and Scott, 1995) (Fig. 11). In relaxed mature tendon, most PG filaments are arranged orthogonally across the collagen fibrils at the gap zone-usually the d band (Scott and Orford, 1981). In immature tendons, PGs are observed either orthogonal or parallel to the D period (Cribb and Scott, 1995) and the amount of PGs associated with collagen fibrils in tendon decreases with increased fibril diameter and age (Scott et al., 1981).

Animal models employing genetic mutations lacking decorin demonstrate collagen fibrils with irregular diameters and decreased skin strength (Danielson et al., 1997) while models lacking lumican show abnormally thick collagen fibrils and skin fragility (Chakravarti et al., 1998). Down regulation of decorin has been shown to lead to development of collagen fibrils with larger diameters and higher ultimate tensile strengths in ligament scar (Nakamura et al., 2000). Models lacking thrombospondin 2, a member of a family of glycoproteins found in extracellular matrix, exhibit abnormally large fibril diameters and skin fragility (Kyriakides et al., 1998). These observations suggest that PGs such as decorin and other glycoproteins found in the extracellular matrix are required for normal collagen fibrillogenesis. Decorin also appears to assist in alignment of collagen molecules in tendon as well as

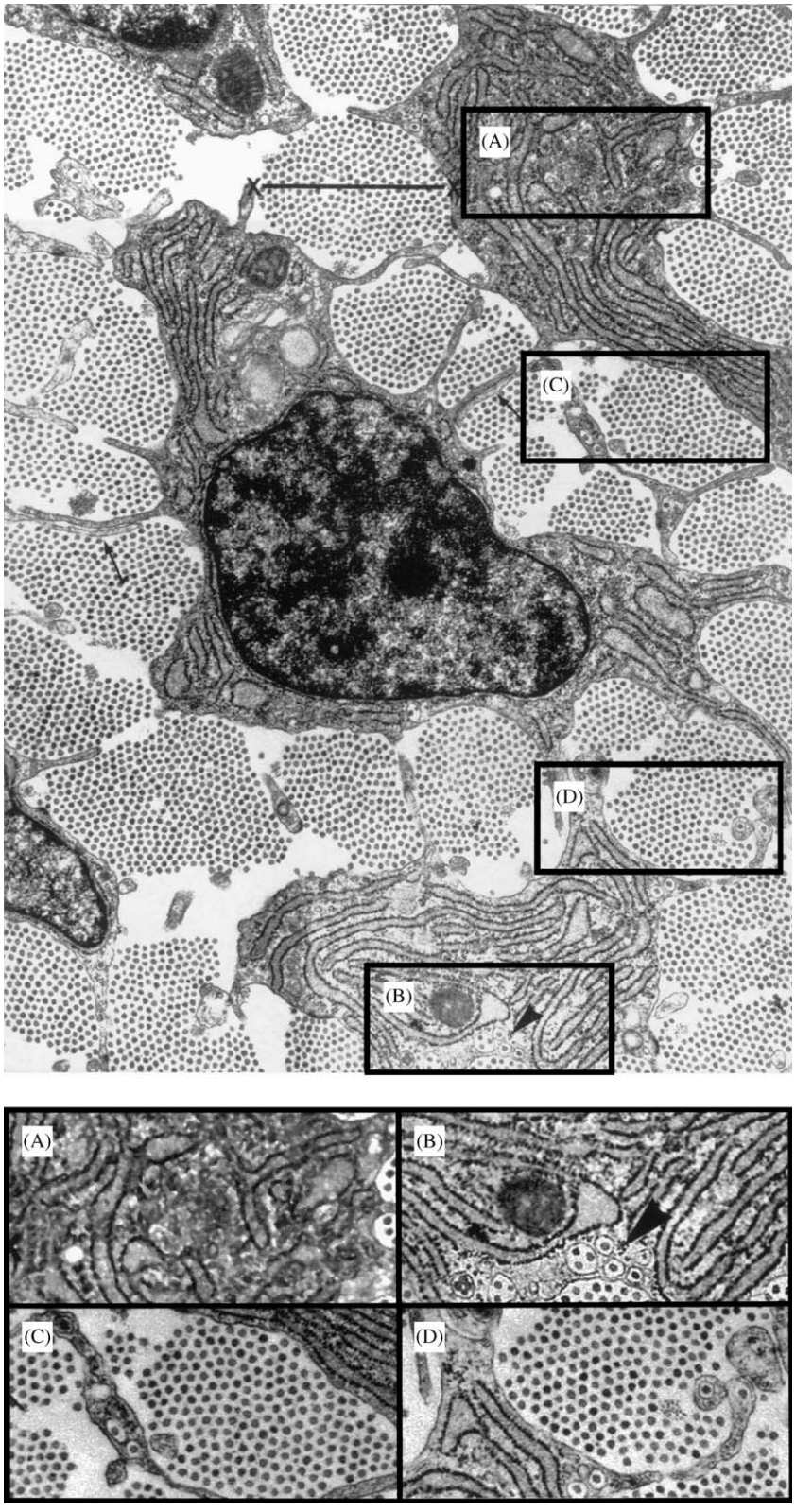

Fig. 8. Addition of axial collagen fibrils within invaginations in the cell membrane to a growing fibril. (Top) Transmission electron micrograph showing collagen fibril formation in invaginations within the cell membrane of a 14-day-old embryo. The arrows are placed in areas of the micrograph where collagen fibrils appear to be in the extracellular matrix and are in close proximity to the cell membrane. Insets A–D show the close relationship between cytoplasmic endoplasmic reticulum and collagen fibrils that appear to be in the extracellular matrix. The middle insets (C and D) show areas where collagen fibrils are within cellular membranes that appear to bud off and add to a growing fibril. The collagen fibril bundle (fiber) diameter marked by two xs is 2 μm. (Bottom) High magnification view of insets shown in top micrograph. These micrographs were adapted from McBride (1984).

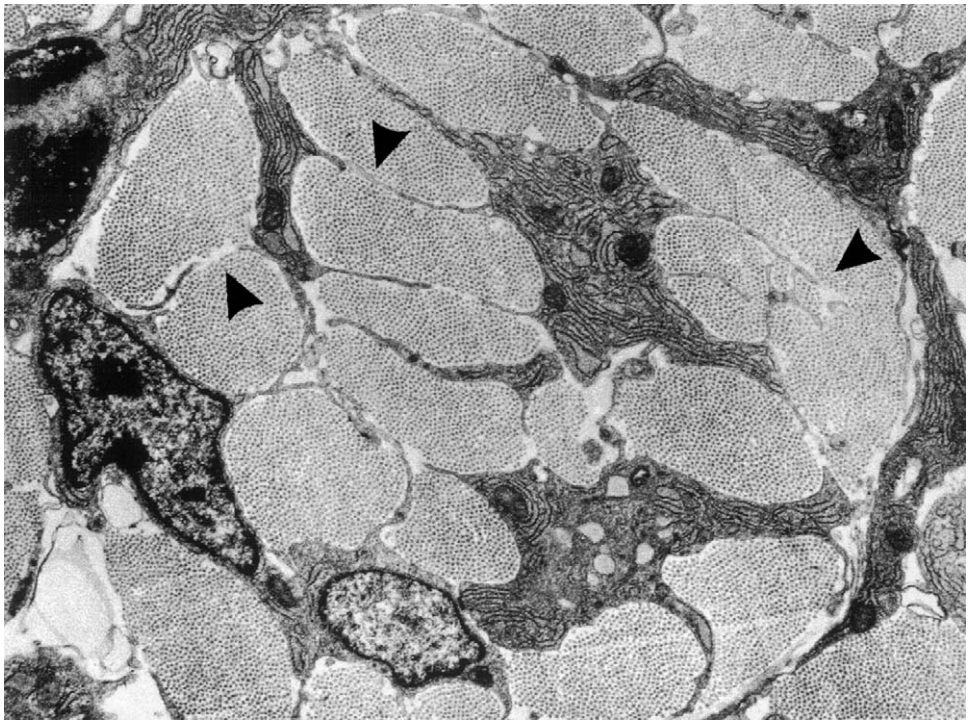

Fig. 9. Lateral fusion of collagen fibrils during fascicle development of chick extensor tendon. Transmission electron micrograph showing the lateral fusion of collagen fibrils at day 17 of chick embryogenesis. Note that the demarcation between collagen fibrils (arrows) is less clear compared to the cross section shown at day 14 (Fig. 8). Several fibrils appear to be in the process of fusion generating fibrils with irregular cross sections. The fibril bundle (fiber) diameter is still about 2 μm before fusion similar to that observed on day 14. This micrograph was adapted from McBride (1984).

facilitates sliding during mechanical deformation (Scott, 1996; Pins et al., 1997).

The role of decorin in development of tendon has been studied by Scott and coworkers (Scott and Orford, 1981; Scott et al., 1981; Scott, 1996) and their results suggest that interactions between collagen and PGs are an important aspect of tendon development. A specific relationship between PGs and the d band of the positive staining pattern of collagen fibrils was observed by Scott and Orford (1981). They speculated that PGs may (1) inhibit collagen fibril radial growth through interference with crosslinking, and (2) inhibit calcification by occupying the hole in the gap zone (Scott and Orford, 1981). Scott and coworkers subsequently demonstrated that during tendon development the interactions between collagen and PGs could be broken down into three phases (Scott et al., 1981). During the first 40 days after conception, collagen synthesis led to the formation of thin fibrils in an environment rich in PGs. Between days 40 and 120, when growth of existing collagen fibrils occurs, PG and hyaluronan content decreased to a critical value. After 120 days, fibril diameter growth decreased and the PG content per fibril surface area remained constant. Recently Scott (1996) has proposed that small PGs act as tissue organizers, orienting and ordering collagen fibrils.

5. Mineralization of tendon

The major leg tendons of the domestic turkey, *Meleagris gallopavo*, including the Achilles or gastrocnemius tendon, begin to naturally calcify when the birds reach about 12 weeks of age (Landis, 1986). This appears to be an adaptation in response to external forces, but the relationship between skeletal changes and such forces is still not understood (Landis et al., 1995). The gastrocnemius is a relatively thick tendon at the rear of the turkey leg which passes through a cartilaginous sheath at the tarsometarsal joint and inserts into the muscles at the hip of the bird (Landis, 1986). After passing through the sheath, the tendon divides into two portions with a decrease in total cross sectional area occurring relative to the original cross section. Mechanically this division results in increased loads borne by the sections after the bifurcation. Initiation of calcification occurs at or near the point of bifurcation and then calcification proceeds along the bifurcated sections (Landis, 1986).

Morphological observations indicate that initiation of calcification occurs on the surface of collagen fibrils close to or at the center of the tendon in 15 week old animals (Nylen et al., 1960). This is associated with changes in the collagen fibril structure. The collagen fibrils appear to become straighter and pack into

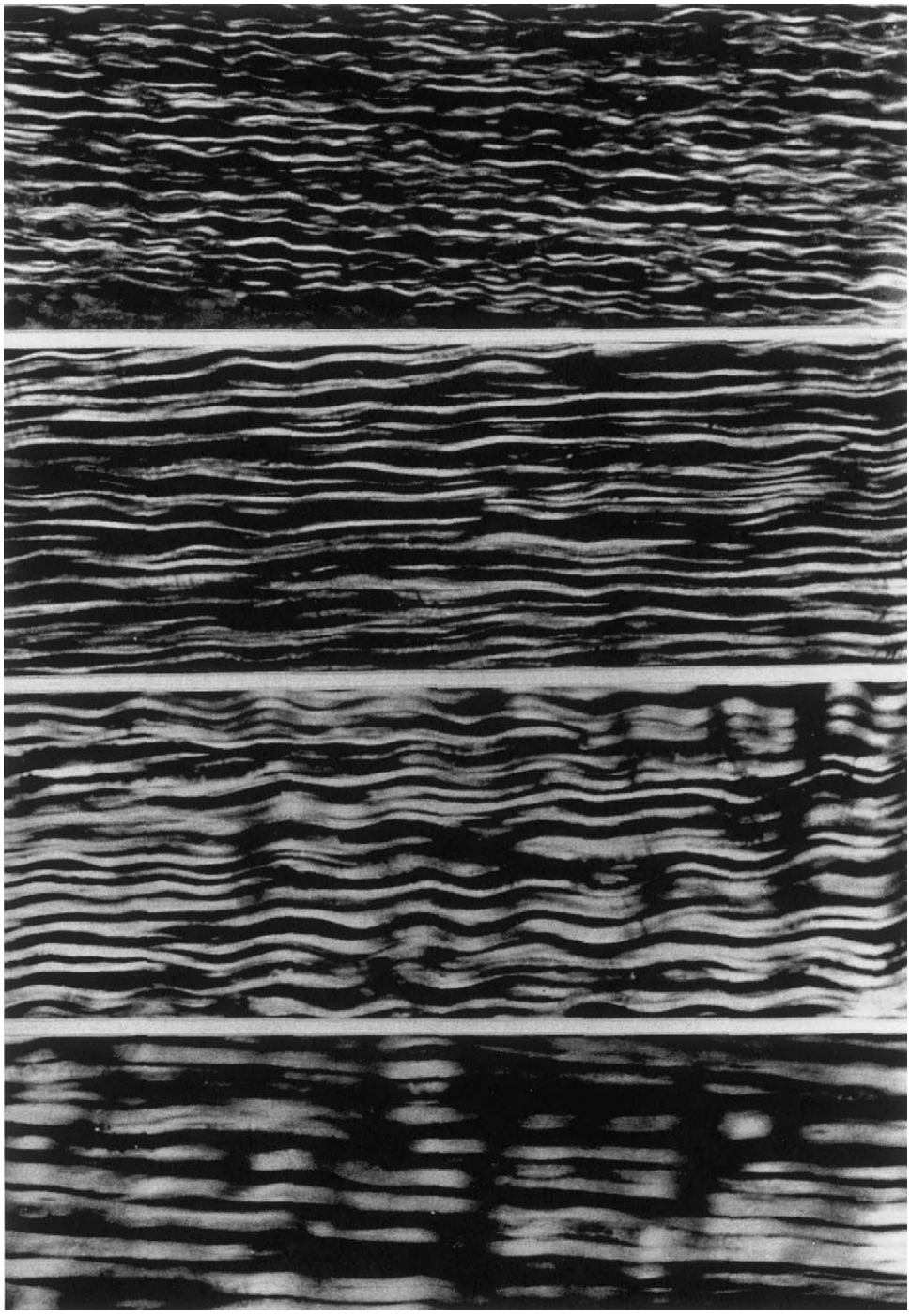

Fig. 10. Polarized-light micrograph of developing chick extensor tendon. This micrograph illustrates how the diameter and apparent length of collagen fibers increases from day 10 (Top) through days 14 and 17 and after 1 day of hatching (Bottom). This micrograph was adapted from McBride (1984).

narrower bundles. Collagen fibrils appear to align with their *D*-periods in register and mineral is laid down within the gap region of the collagen *D*-period (Nylen et al., 1960); the crystal *c*-axis is parallel to the long axis of the fibril. Later mineralization occurs within the fibril.

Studies on calcification of turkey tendons suggest that during the early stages, the hydroxyapatite crystals are about 35.0 nm in length (parallel to the collagen molecule) and 4.0–5.0 nm in width (Engstrom, 1966). X-ray studies showed that the diffraction pattern of uncalcified turkey tendon is similar to that of rat tail tendon (Berthet-Colominas et al., 1979). In the calcifying turkey tendon the mineral in the hole region is crystalline, but that between molecules is amorphous

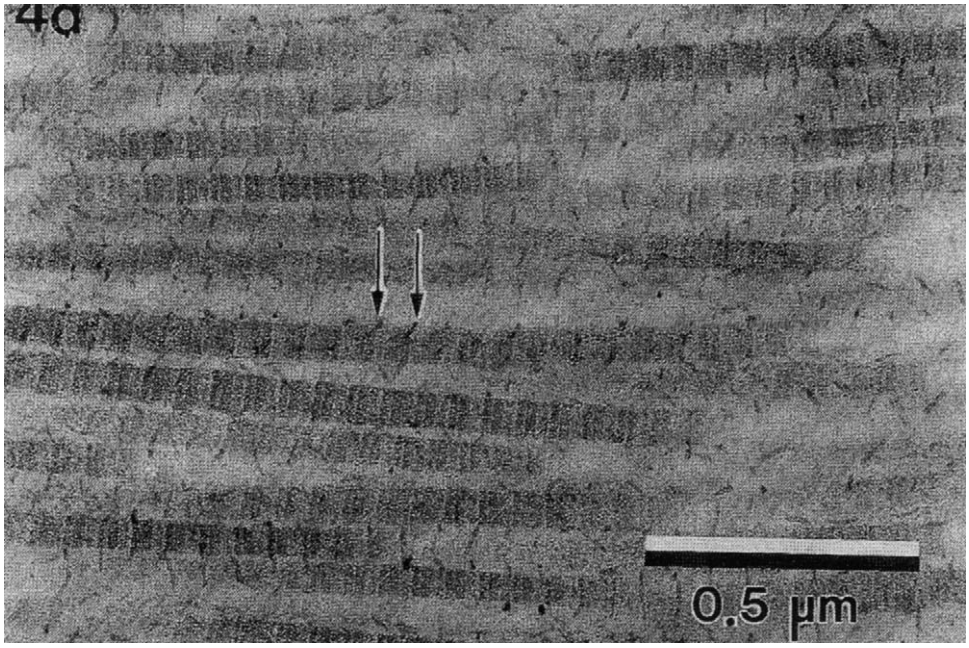

Fig. 11. Relationship between proteoglycans and collagen fibrils in tendon. Transmission electron micrograph showing positive staining pattern of type I collagen fribrils (see Fig. 2) from rabbit Achilles tendon stained with quinolinic blue showing proteoglycan filaments (arrows) attached to collagen fibrils. This micrograph was adapted from Wasserman et al. (1989).

(Berthet-Colominas et al., 1979). Electron micrographs of the mineral show that the crystals are plate-shaped and are arranged in parallel arrays across the collagen fibrils and that the collagen fibrils are elongated in cross section (Traub et al., 1989). Small angle X-ray diffraction of calcified turkey tendon reveals that the inorganic phase is deposited in blocks along the collagen fibrils according to a step function (White et al., 1977). The blocks, which are approximately $0.5D$ long, are regularly arranged along the collagen fibrils with the same 67 nm period as native fibrils. The length of the blocks is independent of the degree of mineralization, however, the height increases as calcification proceeds (Bigi et al., 1988). In an Osteogenic Imperfecta mouse model, the mineral differs in size and alignment with respect to collagen resulting in decreased stress and strain at failure (Misof et al., 1977).

In areas of the tendon away from the site of mineralization, tendon cells are spindle-shaped and have cellular processes that extend into the extracellular matrix and eventually connect with processes of neighboring cells (Landis, 1986). Collagen fibrils range in these areas from 75 to 500 nm in diameter. In regions near the site of mineralization the tendon cells appear to have increased amounts of endoplasmic reticulum, Golgi apparatus and thin cellular processes that weave between tightly packed collagen fibrils (Landis, 1986). Vesicles containing calcium and phosphate are also seen within and outside cellular processes and in regions where mineralization is seen (Landis, 1996).

6. Mechanical properties of developing tendons

The mechanical properties of developing and adult tendons have been studied extensively previously (see Silver et al., 1992 for a review). The purpose of this section is to attempt to relate changes in mechanical properties of tendon to structural changes that are observed at the microscopic and gross levels.

The mechanical properties of developing tendons rapidly change just prior to the onset of locomotion. McBride et al. (1988) report that the ultimate tensile strength (UTS) of developing chick extensor tendons increases from about 2 MPa at 14 days of development to 60 MPa 2 days after birth. This rapid increase in UTS is not associated with changes in fibril diameter, but is associated with increases in collagen fibril lengths (McBride et al., 1988), which can be related to the viscoelastic properties of tendons (Silver et al., 2000b).

The relationship between tendon UTS and fibril length is based on a relationship developed between fibril length and mechanical behavior. Measurements of stress–strain curves and incremental stress–strain curves for tendon and self-assembled collagen fibers suggest that both ultimate tensile strength and the elastic modulus are more dependent on fibril length than diameter (Silver et al., 2000a,b). Incremental stress–strain curves are obtained by applying incremental strains to a tendon and then measuring the initial and equilibrium stresses (Fig. 12). From this plot a "total" stress–strain curve is obtained by plotting the initial

stresses at different strains (Fig. 13). Elastic stress–strain curves are obtained from the equilibrium stresses at different strains. Finally, viscous stress–strain curves are obtained from the difference between the total and elastic stresses at different strains (Fig. 13). Using hydrodynamic theory, shape factors for prolate ellipsoids (Silver, 1987) and assuming isovolumic deformation, the slope of the viscous stress–strain curve is converted into an effective fibril length (Silver et al., 2000a,b). Fibril lengths calculated from incremental stress–strain curves for post-embryonic rat tail and turkey tendons range from about 400 to 800 μm (Silver et al., 2000a,b). These fibril lengths are much greater than the fibril lengths observed prior to the onset of locomotion.

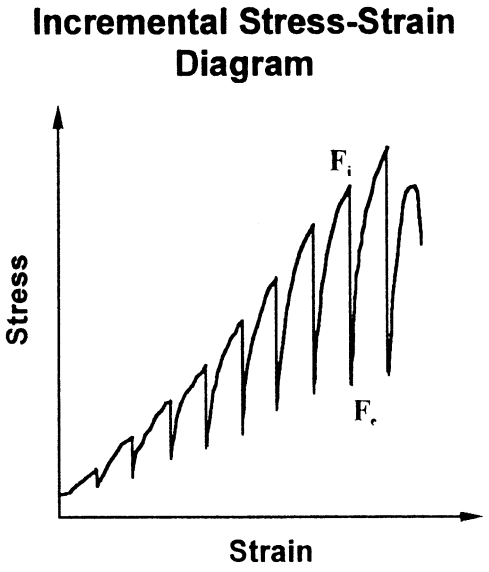

Fig. 12. Incremental stress–strain testing of tendon. Diagram illustrating an incremental stress-versus-strain curve that is obtained by stretching a specimen by a fixed strain increment and then allowing the stress to relax until it equilibrates. At equilibrium an additional strain increment is added and the cycle is repeated until the specimen fails. The total stress is obtained by dividing the total force, F_i, by the cross sectional area while the elastic stress is obtained by dividing, the equilibrium force, F_e, by the cross sectional area. The viscous stress is defined as the difference between the total stress and the elastic stress.

Using mechanical and electron microscopic measurements, fibril lengths have been determined. Effective fibril lengths are obtained from UTS measurements for developing tendons using a calibration curve that correlates tendon UTS values with calculated fibril lengths for self-assembled type I collagen fibers (Fig. 14A). The slope of elastic stress–strain curve is also linearly dependent on fibril length (Fig. 14B). When effective fibril lengths, calculated from mechanical measurements, are plotted against reported values of the fibril lengths measured on chick metatarsal tendons during development (Birk et al., 1989a,b, 1995), a linear relationship is observed. Fig. 15 shows the correlation between the calculated effective fibril length based on mechanical measurements and the actual fibril lengths measured in vivo. The slope of the line is not 1.0; however, variation in the reported values of the fibril segment length in vivo at 14 days of development suggest that it is possible that the slope may be close to 1.0 (Birk et al., 1989a,b, 1995). Considering the data contained in Figs. 14 and 15 together, it appears that the

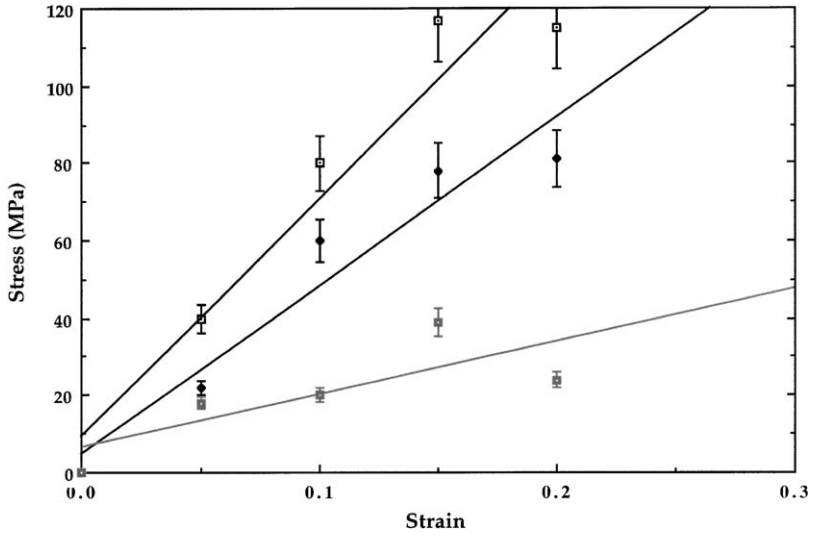

Fig. 13. Total, elastic and viscous stress–strain curves for collagen fibers from rat/tail tendon. The total stress–strain curve (open boxes) was obtained by collecting all the initial, instantaneous, force measurements at increasing time intervals (see Fig. 12) and then dividing by the initial cross sectional area. The elastic stress–strain curve (closed diamonds) was obtained by collecting all the force measurements at equilibrium and then dividing by the initial cross sectional area. The viscous component curve (closed squares) was obtained as the difference between the total and the elastic stresses. Error bars represent one standard deviation of the mean. The data were collected at a strain rate of 10%/min and measurements were made at equilibrium. The time required to reach equilibrium ranged from 20 min to several hours. Diagram reproduced from Silver et al. (2000a).

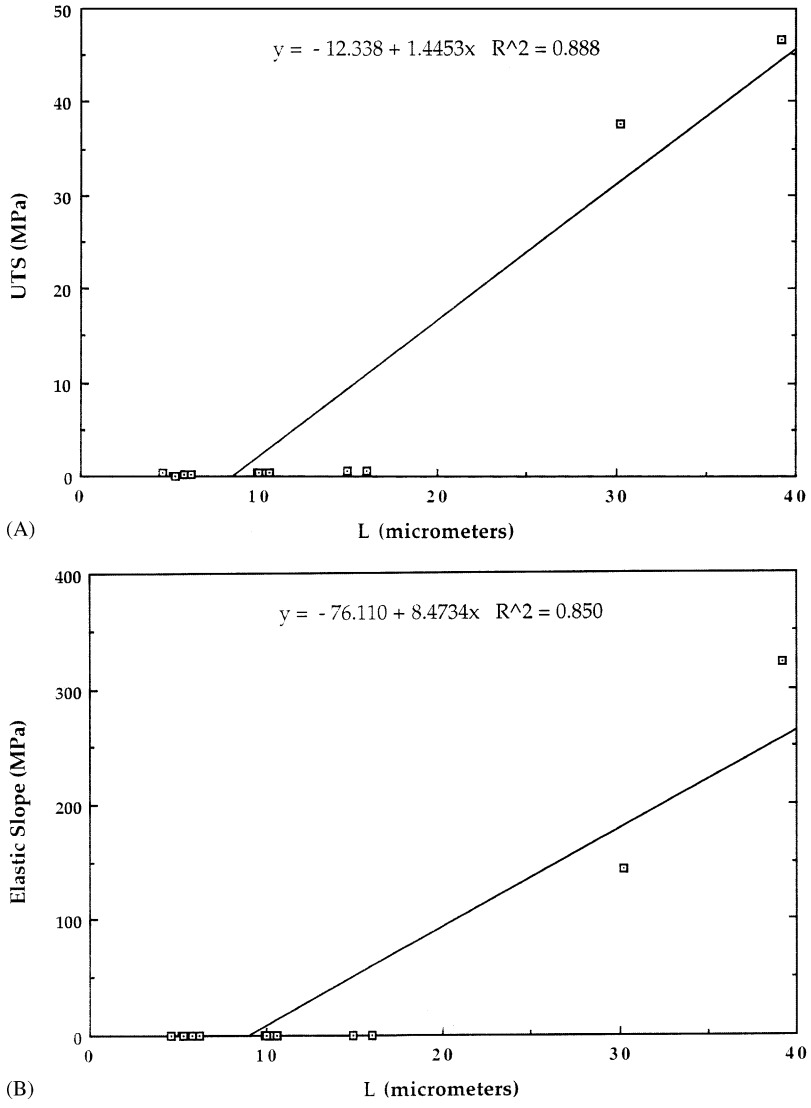

Fig. 14. Relationship between mechanical properties and fibril length (L) for self-assembled collagen fibers. Plot of UTS (A) and elastic slope (B) versus L in μm for self-assembled type I collagen fibers stretched in tension at strain rate of 50%/min. Points with fibril lengths less than 20 μm are for uncrosslinked self-assembled type I collagen fibers while the points above 20 μm are for crosslinked fibers. The correlation coefficient for the best fit line is given by R^2.

increases in UTS that occur during development are associated with increased fibril lengths. This increase in UTS may be a consequence of fibril fusion and crosslink formation in vivo.

On a molecular basis, the slope of the elastic stress–strain curve (elastic modulus) can be related to the stretching of collagen triple helices within crosslinked collagen fibrils (Silver et al., 2000a,b). Studies on self-assembled type I collagen fibers show that in the absence of crosslinks, the elastic slope is reduced (Silver et al., 2000b). Since the elastic slope is also proportional to the effective fibril length (Fig. 14B), the molecular basis for elastic energy storage in tendon appears to involve stretching of collagen triple helices within crosslinked collagen fibrils (see Fig. 3). If collagen is modeled as a composite of flexible and rigid regions then at the molecular level elastic deformation probably results in reversible stretching of the flexible regions of the molecule.

Previous studies have examined the mechanism by which mechanical energy is translated into molecular and fibrillar deformation in tendon (Mosler et al., 1985; Sasakai and Odajima, 1996a,b). Several reports indicate that up to a strain of 2% in tendons, molecular stretching predominates; increases in the collagen D-period beyond 2% occur as a result of molecular slippage (Mosler et al., 1985; Sasakai and Odajima, 1996a,b). The results of other studies suggest that molecular stretching and sliding alone do not explain the elastic and viscous behavior of tendon; the elastic

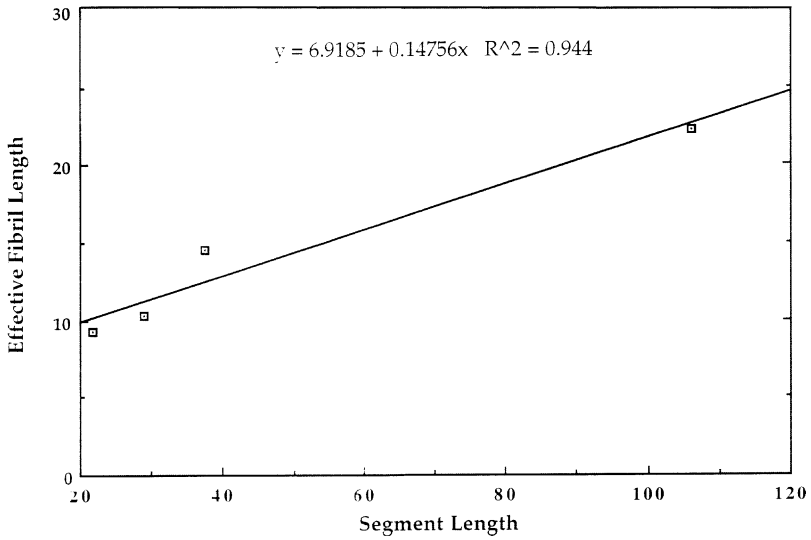

Fig. 15. Effective mechanical fibril length versus fibril segment length. Plot of effective fibril length in μm determined from viscous stress–strain curves for rat tail tendon and self-assembled collagen fibers (Silver et al., 2000a) versus fibril segment length reported by Birk et al. (1995, 1997). The correlation coefficient (R^2) for the line shown is 0.944.

response requires end-to-end crosslinks between collagen molecules within fibrils (Silver et al., 2000a,b). The magnitude of the elastic constant is directly related to the collagen fibril length and therefore the number of collagen molecules that are crosslinked end-to-end in series. The end-to-end crosslink pattern may be specific to tendon and differs from the crosslink pattern in non-skeletal tissues such as skin (Mechanic et al., 1987) suggesting that energy storage in tendon may be different from that in non-skeletal tissues. Although the fibril diameter does play a role in the magnitude of the elastic response, fibril length appears to be the most important parameter in dictating elastic behavior.

The observation that the effective fibril length based on mechanical measurement of viscosity is related to the observed fibril length by microscopy suggests that linear growth of collagen fibrils is key to the development of tendon mechanical stability. This would suggest that early in tendon development, when fibril lengths are short, much of the mechanical energy imparted to the tendon is dissipated through viscous slippage of fibrils less than about 20 μm long. Later when the animal begins to locomote, increases in collagen fibril length beyond 20 μm are associated with increased elastic storage capabilities. A transition from viscous to elastic based mechanical behavior may be a mechanism by which large changes in strength can be achieved rapidly over a short time period without requiring compositional changes in the tissue (Silver et al., 2000a). This could be controlled by the strategic introduction of crosslinks positioned at the ends of collagen molecules to form long fibrils.

Although the role of minor collagen types such as type XII on the mechanical properties of tendon is still unclear, recent evidence suggests that the production of this collagen is high in stretched collagen matrices and suppressed in a relaxed matrix (Chiquet, 1999). Genes for collagen type XII and tenascin-C, another matrix component, contain stretch responsive enhancer regions that upregulate synthesis of these two components as a result of increased mechanical loading (Chiquet, 1999). These observations suggest that changes in external mechanical loading conditions to the extracellular matrix result in synthesis of matrix components that modify the structure and possibly the mechanical properties of tendon.

7. Mechanical properties of mineralizing tendons

The viscoelastic behavior of mineralizing turkey leg tendon has been reported during the period between 12 and 16 weeks (Silver et al., 2000b). Results of these studies suggest that the elastic modulus for type I collagen is between 5 and 7.75 GPa, which is similar to that found for rat tail tendon (Silver et al., 2000b). Fibril lengths obtained from the viscous component of the stress–strain behavior are between 414 and 616 μm, which is slightly smaller than those found for rat tail tendon, but significantly greater than those reported for developing tendons (Birk et al., 1989a,b, 1995; Silver et al., 2000a). These results suggest that mineralization does not change the elastic modulus for type I collagen and the matrix is probably not disassembled and then reassembled during mineralization.

These results are consistent with the "straitjacket" theory proposed by McCutchen (1975) to describe tensile stiffness of bone. McCutchen proposed naturally

kinky collagen molecules in bone were the prime carriers of tension. Mineral crystals deposited around collagen prevent the naturally kinky collagen molecules from straightening under tension, which greatly increases collagen's tensile stiffness without changing its strength. An expanded version of this model would suggest that the mineral deposited in the flexible regions in the collagen molecule (see Fig. 2) would prevent these regions from stretching during tensile deformation by limiting molecular deformation. This would result in no change in the elastic modulus of collagen associated with mineralization, but would cause an increase in the tensile stiffness of mineralizing turkey tendon as has been reported (Landis et al., 1995; Silver et al., 2000b).

8. Collagen self-assembly in vitro

Approximately 50 years ago it was first observed that purified collagen molecules in solution in vitro would spontaneously self-assemble at neutral pH at room temperature to form fibrils that appeared to be identical to those seen in vivo. Self-assembly of collagen to form rigid gels was observed by Gross and coworkers (1952) and Jackson and Fessler (1955). In their experiments, collagen was solubilized and then heated to 37°C in a buffer containing a neutral salt solution. Under these conditions it was recognized that collagen molecules and aggregates of molecules would spontaneously self-assemble forming fibrils that had the characteristic 67 nm repeat distance when viewed in the electron microscope. Subsequent studies showed the addition of ions, alcohols and other substances that affected both electrostatic interactions and hydrophobic bonds were able to modify the assembly of collagen molecules (Silver and Christiansen, 1999). The ability to form fibers from self-assembled collagen fibrils has made it possible to study the properties of model systems that mimic the structure and properties of tendons (Kato et al., 1989; Pins et al., 1997; Silver et al., 2000a).

The mechanism by which collagen molecules self-assemble into fibrils has been a topic of intense research interest since the 1950s. Early studies by Cassel et al. (1962) evaluated the kinetics of the transition of collagen from the solution to the solid phase by raising the temperature over a wide range of pHs and ionic strengths. Their results suggested that collagen self-assembly involved a phase transition with no change in molecular conformation. These workers suggested that it was controlled by the addition of molecules at the fibril surface at temperatures above 16°C, and limited by the rate of diffusion of collagen molecules at temperatures less than 16°C. Thermodynamic studies by Cooper (1970) indicated that native collagen fibril formation was an endothermic process made thermodynamically favorable by the large increase in mobility of the water molecules. This occurred when water molecules and collagen formed separate phases. In contrast to Cooper's study, recent studies have concluded that water mediated hydrogen bonding between polar residues promotes collagen assembly (Leiken et al., 1995).

Early electron microscopic studies suggested that linear growth of fibrils appeared to occur by addition of groups of collagen molecules to form a subfibril; lateral growth occurred by entwining these subfibrils (Trelstad et al., 1976). Interpretation of early studies of collagen self-assembly were confused because of difficulty in obtaining solutions of collagen single molecules as the starting point for self-assembly (Obrink, 1972). Many of the solutions contained aggregates of collagen molecules and therefore interpretation of the results was difficult.

There are several observations that help us understand the process of self-assembly. The first suggests that under typical solution conditions used for preparing soluble collagen, i.e. low pH and low salt content, collagen molecules are in equilibrium with larger aggregates (Yuan and Veis, 1973; Silver and Birk, 1984). The aggregate in equilibrium with single molecules has been estimated to be between 1.5 million (Yuan and Veis, 1973) and 5 million (Silver and Birk, 1984) or between about 5 and 17 molecules. Other studies cited above indicate that the propeptides appear to limit association, thereby limiting the size of the aggregate in equilibrium with single molecules to only 5 molecules (Berg et al., 1986).

Other information suggests that self-assembly of type I collagen leads to the formation of characteristic units that range in length from 4-D staggered dimers (about 570 nm) (Silver et al., 1979; Kobayashi et al., 1985; Ward et al., 1986) to aggregates that are about 700 nm long (Bernengo et al., 1978). This corresponds to between two and three collagen molecules long. Taken together these values of length and width suggest that aggregates formed during the initial phases of self-assembly contain 5–17 molecules and are 2–3 collagen molecules long. Estimates of the diameter of the first aggregates formed are initially 1–2 nm and then 2–6 nm (Gale et al., 1995). These observations are consistent with previously published models that state that self-assembly involves an initial linear step that is followed by both linear and lateral growth steps that occur simultaneously (Silver et al., 1979); reported results are also consistent with the formation of a unit that contains about 5 molecules packed laterally (Christiansen et al., 2000) and is two or three collagen molecules long as diagrammed in Fig. 16. These units appear to grow laterally by fusion since autocorrelation of diameter distributions of self-assembled type I collagen fibrils show a periodicity of about 4 nm (Christiansen et al., 2000).

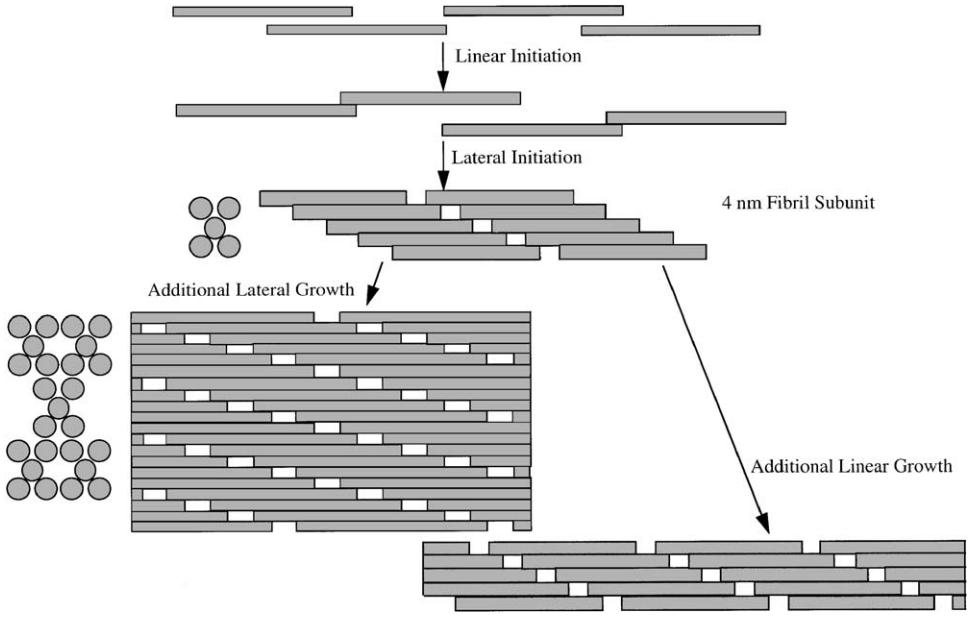

Fig. 16. Model for type I collagen self-assembly. The mechanism for self-assembly of purified type I collagen molecules involves initial linear and lateral growth steps resulting in the formation of 4-D staggered dimers and a fibril subunit with a diameter of about 4 nm. Once the fibril subunit is formed, fibril grow occurs by lateral and longitudinal fusion of 4 nm wide subunits. The rate of linear and lateral growth of fibrillar subunits depends on the solution conditions. Removal of the non-helical ends promotes lateral growth while crosslinking promotes linear growth.

Studies on self-assembly of collagen type I indicate that initiation involves the non-helical end regions (Bensusan and Scanu, 1960), and removal of these ends with enzymes prolongs initiation (Comper and Veis, 1977). Enzymes such as pronase, which cleave both the amino and carboxylic non-helical ends, arrest self-assembly. Pepsin, which removes portions of the amino and carboxylic non-helical ends, slows aggregation resulting in wide fibrils. Leucine aminopeptidase removes the amino terminal non-helical end and leaves the carboxylic end intact causing a lengthened lag phase. Carboxypeptidase A and B remove the carboxyl non-helical end leaving the amino end intact, decreasing lateral growth without affecting the lag phase. From these studies it is concluded that the amino terminal non-helical end is involved in formation of the initial thin fibrillar unit while the carboxy terminal non-helical end is involved in lateral fusion of thin fibrillar units (Comper and Veis, 1977). These conclusions parallel the conclusions made based on self-assembly of procollagen (see Figs. 4 and 16) suggesting the effects of the propeptides and the non-helical telopeptides on type I collagen self-assembly appear to be similar.

One of the draw backs to studying self-assembly using type I collagen is that the fibrils that form are narrow (20–40 nm in diameter) and are not circular. Narrow diameters are likely caused by reactions that occur during self-assembly that favor linear as opposed to lateral growth. Factors such as glycine that block lysine-derived aldehydes during fibrillogenesis increase the width of collagen fibrils (Christiansen et al., 2000). Changes in ionic strength lead to changes in fibril diameter suggesting that lateral growth also involves electrostatic interactions. The non-uniformity and small diameters seen in collagen fibrils self-assembled from collagen molecules suggests that other factors must play a role in controlling fibril fusion and diameter.

Due to limitations associated with studying self-assembly using the collagen molecule, Prockop and coworkers developed a model of collagen self-assembly using selective cleavage of the procollagen I molecule using procollagen proteinases (Miyahara et al., 1982, 1984; Kadler et al., 1987, 1990a,b, 1996). Solubility studies indicate that procollagen and pC-collagen (collagen containing the C propeptide) are both more soluble in solution than pN-collagen (collagen containing the N propeptide) and collagen (Hulmes et al., 1989). Assembly of pN-collagen leads to wide *D*-periodic sheet-like structures (Hulmes et al., 1989). pC-collagen assembles to form tape- and sheet-like structures (Holmes et al., 1991). Incubation of pC-collagen I with C-proteinase processes the pC-collagen to collagen and the collagen assembles into fibrils. Microscopy of the fibrils suggests that they are tightly packed and circular in outline (Hulmes et al., 1989). The average fibril diameter at 29°C is about 650 nm while at 37°C the average diameter is about 150 nm. These diameters are about a factor of ten larger than the diameters of fibrils formed from collagen. These

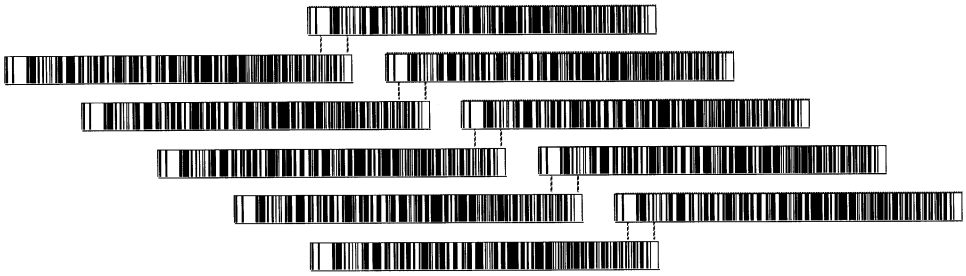

Fig. 17. Collagen crosslinking sites in tendon. Crosslinking sites on collagen occur in the helical and non-helical ends as shown in the diagram. The dotted lines at the ends of the molecule represent the crosslinks that stabilize collagen molecules in tendon. Note the molecules are crosslinked end-to-end and the crosslinks connect rigid regions of the molecule.

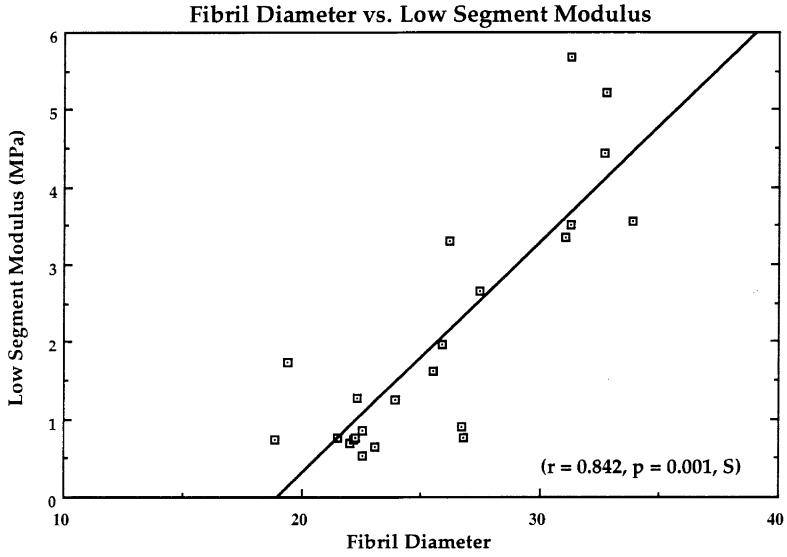

Fig. 18. Relationship between low segment modulus and fibril diameter. Plot of low segment (strain) modulus versus fibril diameter in μm for uncrosslinked self-assembled type I collagen fibrils obtained at a strain rate of 50%/min. Diagram adapted from Christiansen et al. (2000). The correlation coefficient for this plot was found to be 0.842 suggesting that the low strain modulus is related to the fibril diameter.

observations suggest that addition of propeptides allows for formation of large diameter fibrils perhaps by promoting lateral over linear growth. For fibrils self-assembled from procollagen solutions, growth initially occurs at the pointed tip of the fibril; however, later growth occurs at both the pointed and blunt tips (Kadler et al., 1990a).

It is apparent that the ends of the molecule are involved in collagen self-assembly. These ends also overlap with helical parts of the molecule involved in crosslinking (Fig. 17). Veis and George (1994) point out that the crosslinking and collagen cleavage sites have helix stabilities that are different than other parts of the molecule (Veis and George, 1994). They also reported that as the temperature is increased above 10°C, hydrogen bonds which form the major triple-helix stabilizing force become weaker. Weakening of hydrogen bonds within flexible sites on the collagen molecule may promote rotational freedom leading to interactions that support self-assembly.

9. Mechanical properties of self-assembled collagen fibers

Studies on the mechanical properties of self-assembled fibers composed of type I collagen fibrils have provided much insight into the development of matrix mechanical properties. Using this system, changes in parameters such as fibril diameter, length and extent of crosslinking can be correlated with changes in the viscoelastic properties. Danielsen first noted that incubation of self-assembled collagen solutions for periods between 0 and 104 days resulted in a gain in mechanical strength (Danielsen, 1981). He attributed this increase in strength to an increase in the number of crosslinks that form spontaneously over time. Formation of crosslinks within self-assembled collagen fibrils allows one to study the mechanical properties of crosslinked tissue models.

Analysis of the stress–strain behavior of uncrosslinked self-assembled collagen fibers indicates that the initial slope of the stress–strain curve is proportional to the fibril diameter (Christiansen et al., 2000) (Fig. 18). In

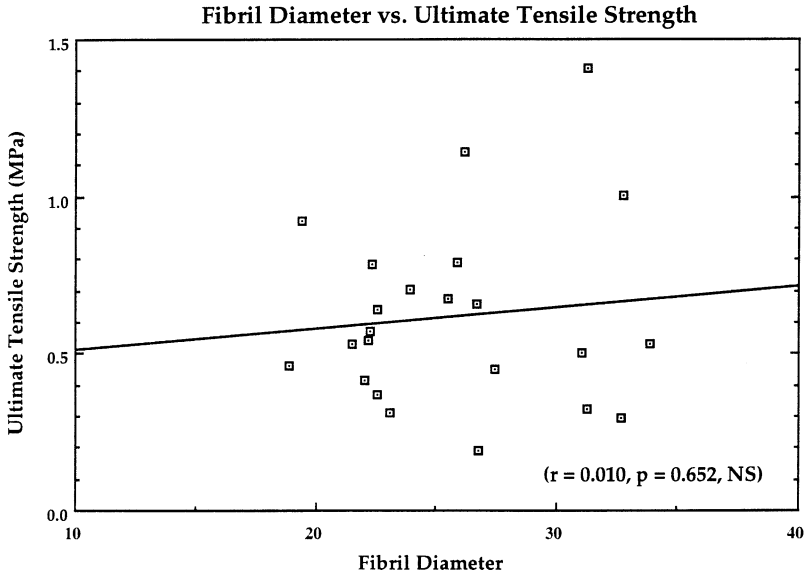

Fig. 19. Relationship between UTS and fibril diameter. Plot of UTS versus fibril diameter in μm for self-assembled type I collagen fibers adapted from Christiansen et al. (2000). The correlation coefficient was found to be 0.010 indicating that fibril diameter is not related to the UTS.

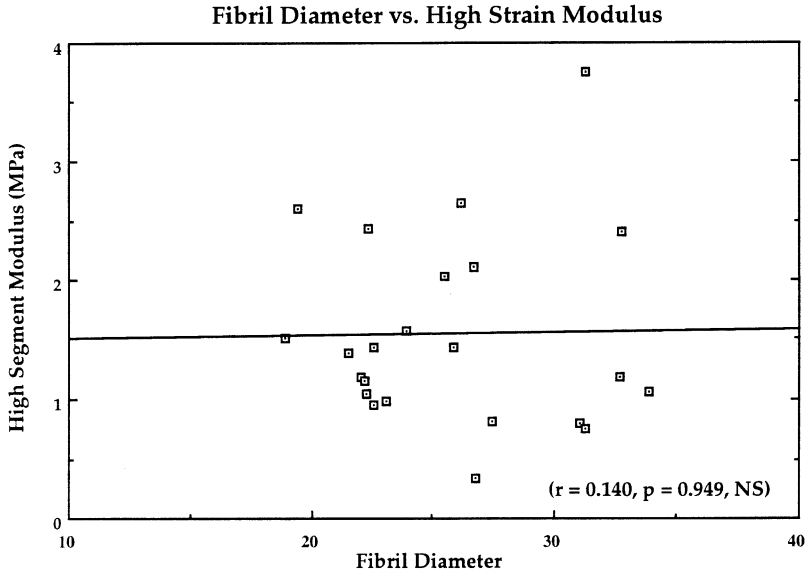

Fig. 20. Relationship between high segment modulus and fibril diameter. Plot of high segment (strain) modulus versus fibril diameter in μm for self-assembled type I collagen fibers adapted from Christiansen et al. (2000). The correlation coefficient was found to be 0.014 indicating that fibril diameter is not related to the high strain modulus.

comparison, the slope in the upper part of the stress–strain curve and the ultimate tensile strength are not correlated with fibril diameter (Figs. 19 and 20). However, values of UTS correlate with the volume fraction of polymer and fibril length for both of these variables (Silver et al., 2000a, 2001a,b). The correlation between UTS and fibril length (Fig. 14A) suggests that the strength of self-assembled collagen and tendon is dependent on the presence of end-to-end crosslinks between molecules.

Measurement of the incremental stress–strain curves for self-assembled collagen fibers have been reported (Silver et al., 2000a). Fibers were stretched in 5% increments until failure and the data was formulated into total, elastic and viscous stress–strain curves (Silver et al., 2000a). The slope of the elastic stress–strain curve, after correction for the volume fraction and the ratio of the macroscopic to microscopic deformation, yielded an elastic modulus of about 6.43 GPa for type I collagen (Silver et al., 2000a). The slope of the viscous stress–strain curve was converted into a fibril length using a prolate ellipsoid model to calculate shape factors and by making the assumption that deformation occurred at constant volume. Results of these studies suggest that the elastic portion of the viscoelastic behavior represents

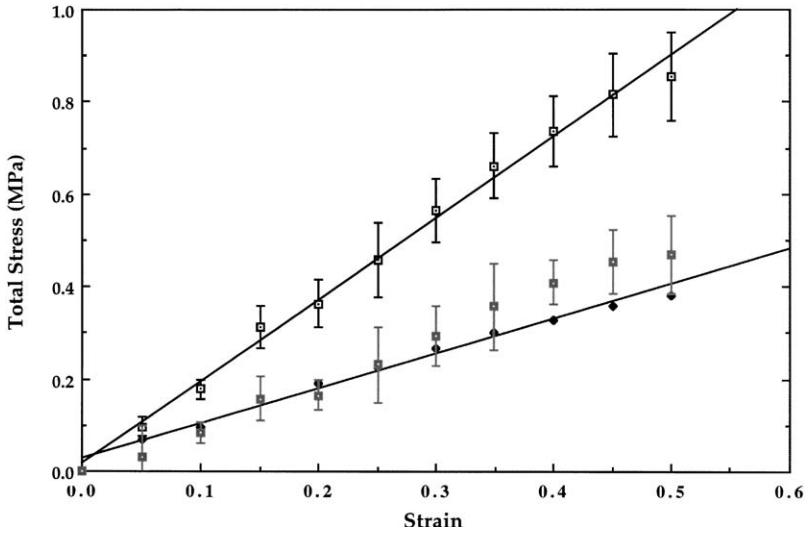

Fig. 21. Total, elastic and viscous stress–strain curves for uncrosslinked self-assembled type I collagen fibers. Total (open squares), elastic (filled diamonds) and viscous (filled squares) stress–strain curves for self-assembled uncrosslinked collagen fibers obtained from incremental stress–strain measurements at a strain rate of 10%/min. The fibers were tested immediately after manufacture and were not aged at room temperature. Error bars represent one standard deviation of the mean value for total and viscous stress components. Standard deviations for the elastic stress components are similar to those shown for the total stress but are omitted to present a clearer plot. The straight line for the elastic stress–strain curve closely overlaps the line for the viscous stress–strain curve. Note that the viscous stress–strain curve is above the elastic curve suggesting that viscous sliding is the predominant energy absorbing mechanism for uncrosslinked collagen fibers. This figure was adapted from Silver et al. (2000a).

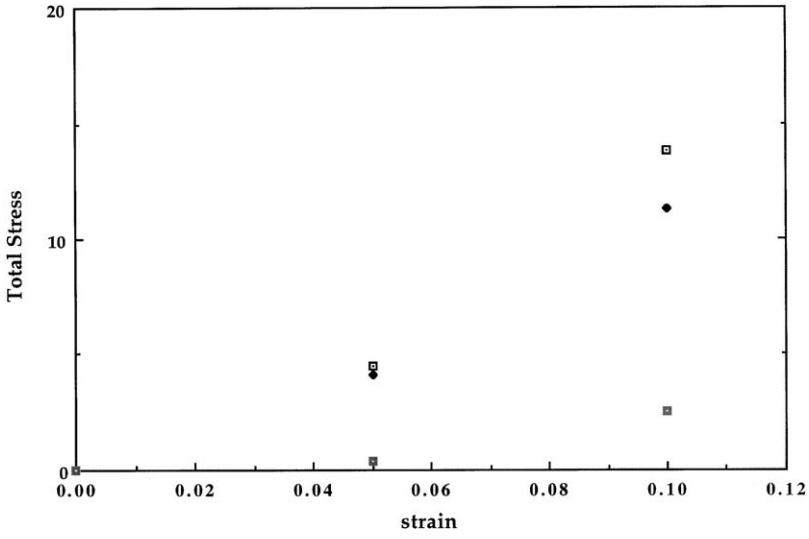

Fig. 22. Total, elastic and viscous stress–strain curves for crosslinked self-assembled collagen fibers. Total (open squares), elastic (filled diamonds) and viscous (filled squares) stress–strain curves for self-assembled collagen fibers obtained from incremental stress–strain measurements at a strain rate of 10%/min. The fibers were tested after aging for 3 months at room temperature and pressure. Note that the viscous stress–strain curve is below the elastic curve suggesting that elastic deformation is the predominant energy absorbing mechanism for crosslinked collagen fibers. This figure was adapted from Silver et al. (2000a).

stretching of the collagen triple helix. In the absence of crosslinks between collagen molecules the elastic portion is smaller than the viscous one (Silver et al., 2000a) (Fig. 21). The introduction of crosslinks increases the elastic portion of the viscoelastic behavior (Fig. 22). The viscous portion reflects the viscous slippage of collagen fibrils past each other which is decreased by the formation of crosslinks. These observations suggest that crosslinking is an efficient means of controlling the mechanical behavior of collagen fibrils; introduction of crosslinks increases the UTS and elastic contribution to the stress–strain behavior.

The strain rate dependence of the elastic and viscous stress–strain curves has been studied for self-assembled collagen fibers (Silver et al., 2002b). The slope of the elastic stress–strain curve was reported to be strain rate independent at strain rates between 10%/min and 1000%/min (Silver et al., 2002b). In contrast the slope

of the viscous stress–strain curve was found to decrease after correction for the strain rate suggesting that at high strain rates collagen fibrils "hydroplane" by each other reducing the apparent viscosity (Silver et al., 2002b).

10. Effects of physical forces on cell–extracellular matrix interactions

We have implied in this paper that the mechanical properties of tendon are to a first approximation due to the behavior of the collagen fibril network; this is an oversimplification. The mechanical properties of tendon are dynamically dependent on the properties of the crosslinked collagenous network and also on cell–extracellular matrix interactions. It has been hypothesized that forces exerted by the extracellular matrix on cells may be in equilibrium with forces exerted by cells on the extracellular matrix (Ingber, 1991, 1999). Ingber (1991) proposed that forces are transmitted to and from cells through the extracellular matrix with changes in mechanical forces and cell shape acting as biological regulators. He further hypothesized that cells use a tension-dependent form of architecture, termed tensegrity, to organize and stabilize their cytoskeleton. Mechanical interactions between cells and their extracellular matrix appear to play a critical role in cell regulation by switching cells between different gene products.

Integrin adhesion receptors that connect extracellular matrix components and cytoskeletal elements have been implicated in mediating signal transduction through the cell membrane in both directions (Liu et al., 2000). Integrin adhesion receptors are heterodimers of two different subunits termed α and β (Liu et al., 2000). They contain a large extracellular matrix domain responsible for binding to substrates, a single transmembrane domain and a cytoplasmic domain that in most cases consists of 20–70 amino acid residues (Hynes, 1992). They mediate signal transduction through the cell membrane in both directions: Binding of ligands to integrins transmits signals into the cell and results in cytoskeletal reorganization, gene expression and cellular differentiation (outside-in signaling); on the other side signals within the cell can also propagate through integrins and regulate integrin–ligand binding affinity and cell adhesion (inside-out signaling) (Hynes, 1992; Schwartz et al., 1995).

Eukaryotic cells directly attach to extracellular matrix collagen fibers via integrin subunits $\alpha1\beta1$ and $\alpha1\beta2$ (Xu et al., 2000) through a six-residue sequence (glycine–phenylalanine–hydroxyproline–glycine–glutamic acid–arginine) sequence (Knight et al., 2000) that is present in the b2 band of the collagen positive staining band (Fig. 2). Integrins are transmembrane molecules that associate via their cytoplasmic domains with a number of cytoplasmic proteins including vinculin, paxillin, tensin and others (Yamada and Geiger, 1997), which are all involved in the dynamic association with actin filaments. In cultured cells, integrin-based molecular complexes form small (0.5–1 μm) or point contacts known as focal complexes (Nobes and Hall, 1995) and elongated streak-like structures (3–10 μm long). The elongated structures are associated with actin and myosin containing filament bundles (stress fibers) known as focal contacts or focal adhesions (Heath and Dunn, 1978). Results of recent studies suggest that integrin-containing focal complexes behave as mechanosensors exhibiting directional assembly in response to local force (Riveline et al., 2001). It has been reported that collagen binding integrins are involved in down-regulating collagen $\alpha1$(I) and upregulating collagenase (MMP1) mRNA when fibroblasts are grown in a relaxed collagen gel (Langholz et al., 1995).

Effects of mechanical forces have been studied on isolated fibroblasts and fibroblasts cultured in a collagen matrix. Fibroblasts cultured on flexible-bottom surfaces coated with fibronectin, laminin or elastin were observed to align perpendicular to the force vector (Breen, 2000). Mechanically loaded cells grown on laminin or elastin or other substrates expressed higher levels of procollagen mRNA and incorporated more labeled proline into protein than did unstressed cells (Breen, 2000). Fibroblasts in cell culture that are not aligned with the force direction show a several-fold increase in matrix metalloproteinase activity (MMP1, MMP2 and MMP3) suggesting that cells that are unable to align with the direction of the applied load remodel their matrix more rapidly than oriented cells (Mudera et al., 2000).

Fibroblasts grown in a three-dimensional collagen lattice have been shown to align themselves with the direction of principle strain (Eastwood et al., 1998a,b) and adopt a synthetic fibroblast phenotype characterized by induction of connective tissue synthesis and inhibition of matrix degradation (Kessler et al., 2001). Fibroblasts grown in collagen lattices can generate a force of approximately 10 N as a result of a change in cell shape and attachment (Eastwood et al., 1998a,b). Fibroblasts have the ability to maintain a tensional homeostasis of approximately $40–60 \times 10^{-5}$ N per million cells (Eastwood, 1998). Cell contraction of 3-D collagen matrices was observed to be opposite to the direction of applied loads (Brown et al., 1998). Increased external loading was followed immediately by a reduction in cell-mediated contraction (Brown et al., 1998).

Tenacin-C is an extracelluar matrix protein that contributes to the mechanical stability of extracellular matrix through its interaction with collagen fibrils; the interaction appears to be mediated by decorin (Elefteriou et al., 2001). Type XII collagen is a fibril-associated collagen with an interrupted triple helix (FACIT collagen) that is in close association with type 1 collagen fibrils in tissues (Keene et al., 1991). In matrices bearing

high mechanical loads, expression of mRNA and synthesis of tenacin-C and collagen XII are upregulated (Karimbux and Nishiura, 1995; Webb et al., 1997). Stretch response promoter regions have been identified in tenascin-C and collagen type XII genes. These regions have the same motif as has been implicated in the response of endothelial cells to platelet derived growth factor (PDGF-B) under the influence of shear stress (Resnick et al., 1993; Khachigian et al., 1995).

11. Summary

Collagen assembly in developing tissues is controlled by the synthesis of procollagen within the cell, deposition of procollagen in extracytoplasmic compartments, cleavage of the N and C propeptides extracellularly, and lateral fusion of fibril intermediates that results in linear and lateral growth of fibrils. All of these processes can occur in the absence of crosslinks between collagen molecules; however, the development of strength required for locomotion by skeletal tissues requires the conversion of highly viscous liquid-like assemblies of collagenous macromolecules into solid tissues that can store elastic energy. The elastic storage of energy in tendons during locomotion appears to principally involve the sequential stretching of flexible segments within the collagen triple helix. For this to occur efficiently the molecules must be crosslinked end-to-end within a fibril. Elastic energy storage during deformation of integrin binding regions found in collagen fibrils may stimulate stretch responder promoter regions in fibroblasts that stimulate expression of macromolecules of the extracellular matrix and result in up regulation of collagen synthesis and down regulation of collagenase.

Rapid changes in the mechanical properties of developing skeletal tissues can be explained by the crosslinking of networks of collagen fibrils in embryonic tissues and is consistent with the hypothesis that early in development, viscous sliding of fibrils plays an important role in the mechanical response of animal tissues to forces experienced in utero. Later in development when locomotion is required, mechanical stability is primarily a result of elastic deformation of the flexible parts of the collagen molecule within crosslinked fibrils. Viscous losses occur by the slippage of collagen fibrils past each other during deformation. It is our hope that by understanding the molecular and fibrillar basis of energy storage and dissipation in tendons it may be possible to develop new insight into what roles mechanical forces play in biological systems.

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