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Review

Assembly of the tendon extracellular matrix during development*

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ABSTRACT

The assembly of the collagenous extracellular matrix during tendon development was studied to determine the mechanisms involved in collagen fibril growth. Developing avian metatarsal tendons were studied using structural, immunochemical and biochemical approaches. Tendon fibroblasts were shown to establish a hierarchy of extracellular compartments associated with fibrils, bundles, and macroaggregates during development. These distinct domains provide a mechanism for the fibroblast to influence the extracellular steps in matrix assembly. A discontinuous fibrillar matrix was assembled and fibril segments ~ 10–20 μm long were deposited into bundles by the 14 d embryo fibroblasts. The fibril segment is a normal assembly intermediate, permitting orderly linear, lateral and intercalatory development and growth. A lateral and/or linear fusion of segments may be responsible for the formation of mature continuous fibrils. Fibril segments were isolated from 12–18 d chick embryo metatarsal tendons. Homogenisation almost completely disrupted the 12–15 d tendons. Transmission electron microscopy demonstrated intact segments. Between d 12 and 15 of development, mean segment length increased from ~ 22 to 33 μm. The incremental increase in length with development indicates a limited linear fusion of segments which is supported by morphological examination. At 16 d, there was a significant decrease in segment extractability and by 17–18 d, intact segments were unextractable. Mean segment lengths were 37 μm and > 70 μm for 16 and 17 d tendons respectively. During this period, fibril diameter also increased. These findings demonstrate both lateral and linear fusion of segments, also supported by morphological data, and are consistent with in situ data, indicating an increase in fibril length between 14 and 18 d of development. The postdepositional lateral and/or linear fusion of fibril segments are important processes allowing for orderly development and growth.

Key words: Chick embryo, collagen, fibroblasts.

INTRODUCTION

Tendon is a uniaxial connective tissue which connects muscle to bone and serves to transmit force. Tendons are composed of highly aligned collagen fibrils, collected into bundles. The fibril bundles together with the tendon fibroblasts are organised into fascicles, and fascicles are bound together in a connective tissue sheath to form a tendon. The orderly assembly and maintenance of this hierarchy are required for normal development as well as the establishment of mechanical integrity and function (Birk & Trelstad, 1986; Birk et al. 1989*a*, *b*, 1991).

Attention has focused on the process of collagen fibril formation, although it is not the fibril itself which represents the major morphogenetic unit in most tissues, but groups of fibrils organised into bundles (Birk & Trelstad, 1984, 1986; Trelstad & Birk, 1984). These units are interwoven in specific patterns which define tissues. Elucidation of the mechanisms regulating fibril assembly, deposition and growth is necessary for understanding the main-

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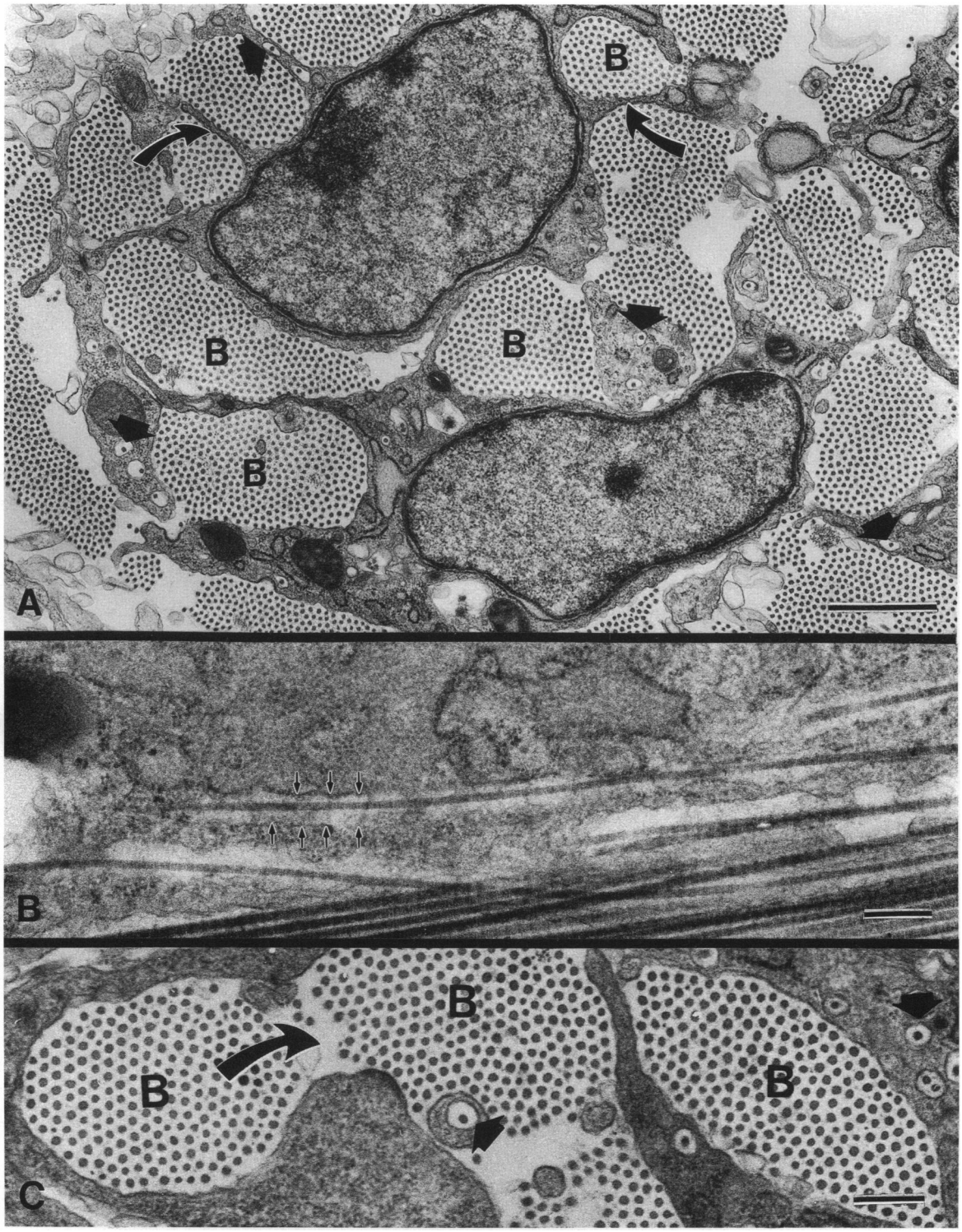

Fig. 1. Compartmentalisation of the extracellular matrix during tendon development. Transmission electron micrographs illustrating the complexity of the fibroblast surface and the partitioning of the extracellular space with formation of extracellular compartments in tendon. A section cut perpendicular to the tendon axis in a 14 d chick embryo (panel *A*) shows the fibril-forming channels (arrowhead), the bundle-forming spaces (B) and the macroaggregate-forming spaces which form as cytoplasmic processes separating bundle-forming compartments retract (curved arrows) and bundles coalesce to form macroaggregates. A section cut parallel to the axes of 14 d chick embryo tendon illustrates a narrow cytoplasmic channel (panel *B*). This channel has periodic indentations (small arrows) of the plasma membrane, indicative of its formation from elongate secretory vacuoles, and contains a collagen fibril. In panel *C*, cross sections of fibril formation channels are illustrated (arrowheads). Sections 200–250 nm thick. Bars, 1 μm (*A*), 500 nm (*B*, *C*).

tenance of structure and tissue function during development, appositional and intercalatory growth as well as repair. Understanding how collagen fibrils become organised into tissue-specific structures with unique spatial patterns is a central issue in morphogenesis.

FIBRIL FORMATION INVOLVES A HIERARCHY OF EXTRACELLULAR COMPARTMENTS

During tendon development, fibroblasts define at least 3 distinct extracellular compartments and these complex specialisations of the fibroblast cell surface are important for at least 3 different levels of matrix organisation: collagen fibrils or fibril segments, fibril bundles and tissue specific macroaggregates such as the large bundles in the fascicles of the tendon. The hierarchy of extracytoplasmic compartments is created by the fibroblast during tendon morphogenesis and represents an extension of the cell's domain into the extracellular space. In the developing tendon, fibril assembly occurs in close association with the cell surface. The tendon fibroblast compartmentalises the extracellular space to form defined regions involved in fibril, bundle and macroaggregate formation (Trelstad & Hayashi, 1979; Trelstad & Birk, 1984; Birk & Trelstad, 1986; Birk et al. 1990, 1991). The complexity of the fibroblast surface is evident in sections cut perpendicular and parallel to the axes of d 14 (stage 40) chicken embryo tendons (Fig. 1). The first compartment consists of a series of narrow channels containing single or sometimes small groups (2 or 3) of fibrils. These fibrils are enveloped by the fibroblast membrane and course deep within the cytoplasm. Serial sections reveal that these narrow channels originate deep within the cytoplasm often in a perinuclear position associated with the Golgi region and distally are open to the extracellular space. These narrow, deep channels fuse with each other laterally and add to the 2nd fibroblast delimited compartment where fibrils coalesce to form bundles. The 2nd extracellular compartment consists of fibrils grouped as fibril bundles in close association with the cell surface. The 2nd compartment is defined by a single fibroblast or sometimes by adjacent fibroblasts. It is within this compartment that fibrils coalesce to form fibril bundles. The 3rd compartment consists of laterally associated fibril bundles (macroaggregates). This compartment is defined by the apposition of 2 or 3 adjacent fibroblasts. The tendon macroaggregates which form in this compartment by the lateral association and coalescence of fibril bundles are partially surrounded by interdigitating processes. An analogous compartmentalisation of the extracellular space is seen in the developing cornea (Birk & Trelstad, 1984) and dermis (Ploetz et al. 1991).

This hierarchy of compartments provides a series of controlled spaces in which the extracellular events in collagen fibrillogenesis and hierarchical development of tissue architecture can occur sequentially. The compartmentalisation permits the cell to partition the forming collagen fibrils, fibril bundles and aggregates as well as the processing enzymes necessary for the postdepositional modifications of collagen molecules, other matrix components and collagen binding proteins which may be important in the interactions of collagen with the cell surface. The possibility of subtle control of the microenvironment permits the cell to alter the environment to promote/inhibit or modify a particular postdepositional event or the interaction with specific macromolecules at a particular time during fibrillogenesis. The formation of distinct compartments in which the structural elements of the developing matrix form also would serve to position these elements physically within the developing matrix.

DURING TENDON DEVELOPMENT THE COLLAGENOUS MATRIX IS ASSEMBLED FROM FIBRIL SEGMENTS

Tendon fibroblasts in 14 d chicken embryos produce fibrils as discontinuous segments. Fibril segments are assembled within the extracytoplasmic channels and then added to a fibril bundle containing other segments (Birk et al. 1989*a*). The length and diameters of the fibril segments in bundles are determined from serial sections. The ends are recognisable by their significantly smaller diameters as well as by their disappearance in the serial section set and the lengths varied from 7–15 μm (Birk et al. 1989*a*, 1990). The fibril segments are asymmetric, consistent with work on fibril growth in vitro (Holmes & Chapman, 1979; Kadler et al. 1990).

The in situ data indicate that fibril segments are intermediates in fibril assembly and that such segments are maintained in the tendon during the initial stages of development. This being the case, these discrete fibril segments should be extractable from tissues. This hypothesis was confirmed by examining extracts from 14 d embryonic metatarsal tendons (Fig. 2). Electron microscopy of the negatively stained homogenate demonstrated the presence of intact fibrils. Tendons were removed, washed in phosphate-buffered saline (PBS) and swollen at 4 °C in PBS pH 7.3 with protease inhibitors followed by gentle

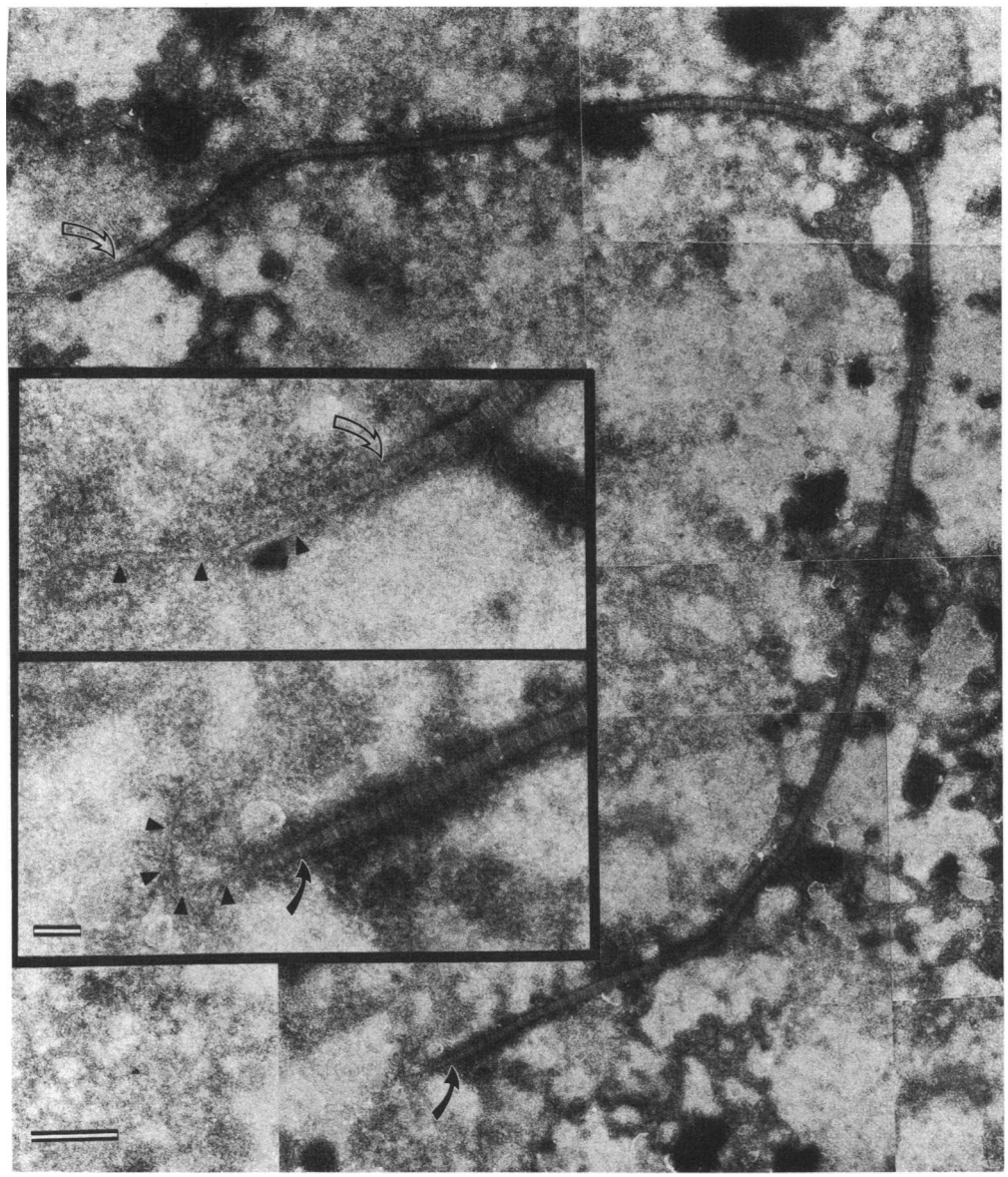

Fig. 2. Structure of collagen fibril segments. Fibril segments were extracted from 14 d chick tendons. Tendons were washed, swollen and homogenised. This procedure almost completely disrupted the 14 d tendons. The suspension was negatively stained and observed by transmission electron microscopy. Intact fibrils of discrete lengths were observed and an example is presented. The ends of extracted fibril segments were asymmetric as described in situ, with a long and short tapers (inset). Bar, 500 nm; bar (inset) 100 nm.

homogenisation with a Donce homogeniser. This procedure almost completely disrupted the 14 d tendons. The suspension was centrifuged and the supernatant negatively stained and observed by transmission electron microscopy. The supernatant contained intact fibrils of discrete length. We have measured 25 fibrils from 14–15 d chicken embryo tendon and found a mean length of ~ 30 μm (range 12–82 μm) most (16) in the 17 to 35 μm range. The fibril ends were asymmetric as described in situ, with a short and long taper (Fig. 3). Only fibrils with clearly identified, intact ends were measured. Broken ends were easily recognised and not common at this stage of development. The observed lengths for segments extracted from 14 d tendons were longer than those measured in vivo from 14 d tendons (Birk et al. 1989*a*). However, these data were consistent with the presence of fibril segments. There are a number of possible explanations for this increased length, but it most likely reflects the in situ reconstructions being assembled from limited data sets which may select for shorter segment that may be newly deposited and less mature.

FIBRIL SEGMENTS MATURE TO FORM CONTINUOUS FIBRILS

We predict that as development proceeds there is a lateral and/or linear fusion of fibril segments to form mature, more continuous fibrils. This hypothesis

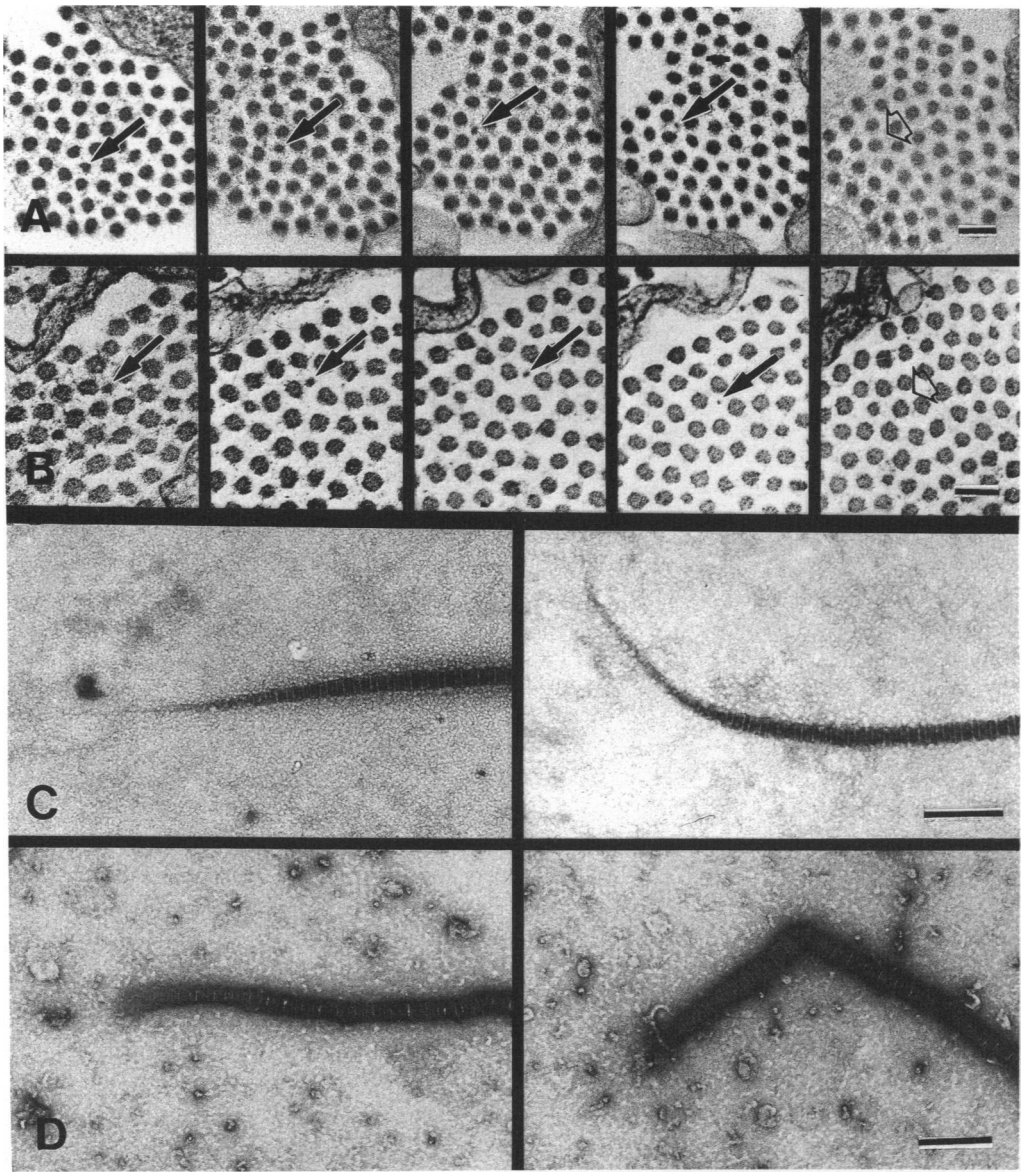

Fig. 3. Fibril segments, and consequently fibril bundles, are discontinuous. Serial 200 nm thick sections were cut perpendicular to collagen bundles in the chick embryos from (*A*) 14 d tendon and (*B*) 17 d tendon. The serial transmission electron micrographs demonstrate the ends of collagen fibrils within fibril bundles (arrows). In (*A*) and (*B*) the fibril decreases in diameter from left to right. Not all plates are presented as consecutive sections because of space limitations, but all are prepared from serial data sets. Fibril segments can be extracted from tissues during development. Tendons were washed, swollen and homogenised. (*C*) The 14 d tendons were almost completely disrupted, yielding segments with short (left column) and long (right column) tapered ends. (*D*) The 17 d tendons were unextractable, yielding fibril fragments with broken ends. Bars, 100 nm (*A*, *B*), 300 nm (*C*, *D*).

predicts a change in fibril segment diameter and/or length with maturation. The analysis of serial 150 nm thick sections through ~ 50 μm of the 18 d chicken embryo tendon demonstrated that the ends of fibrils were still readily identified both within fibril-forming channels as well as within fibril bundles. Examination of these older tendons demonstrated the newly formed fibril segments within channels had a length similar to that at 14 d of development.

Consistent with a fusion of fibril segments having occurred between 14 and 18 d of development, when 17–18 d chicken embryo tendons are homogenised very little disruption of the tendons is observed. The number of intact fibril segments is significantly reduced with only 3 complete fibril segments having been isolated. These had lengths of ~ 89, 117 and 170 μm. This extraction procedure for 17–18 d tendons produced predominantly fibrils with broken ends (Fig. 3). These fragments were long and indicative of a significantly increased fibril length. Thus, as development proceeds, there appears to be a linear and/or lateral fusion of fibril segments to form

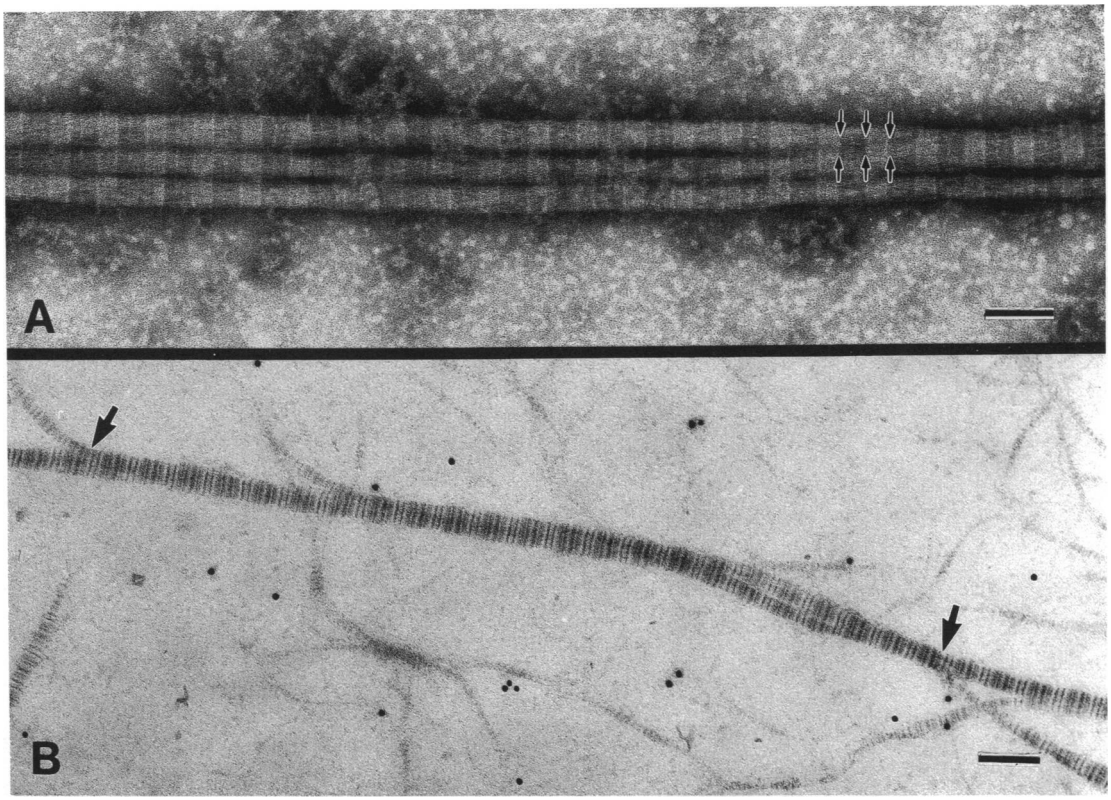

Fig. 4. Collagen fibrils grow by lateral associations of fibril segments. Transmission electron microscopy of fibrils from extracted (*A*) and cryosectioned (*B*) tendons illustrating lateral association of fibril segments. The extensive lateral association/fusion of fibril segments would produce fibrils of increasing length and larger diameter. Bar, 100 nm.

mature, more continuous fibrils which by 18 d of development are too long to extract. During this period the fibril diameters also increase with the generation of a multimodal distribution, consistent with lateral fusion of fibril segments.

Trotter & Wolfsky (1989) presented morphological data from the mature rat tendon indicating that fibril–fibril fusions occur. Scott (1984) proposed that a fusion of fibrils occurs, with possible regulation via a surface coat of decorin (dermatan sulphate proteoglycan). During chicken tendon development, fibril segment length increases incrementally between d 10 and 16 of tendon development. Preliminary analysis indicates that this increase in mean segment length is due to the addition of a population of longer segments rather than a shift of the entire population. Morphological analysis of fibrils (segments) in situ and extracted from tissues demonstrated the lateral association of fibril ends (Fig. 4). The interactions most frequently observed during d 12–15 of tendon development were lateral associations (end-to-end) of adjacent tapered segment ends with a maintenance of fibril polarity. This type of interaction would yield a population of longer fibril segments with a minimum increase in fibril diameter. There is an abrupt increase in mean segment length beginning at 17 d of development as determined from measurements of extracted fibril segments from metatarsal tendons at different stages of development. These observations were entirely consistent with the in situ observations from 18 d tendons indicating an increase in length between 14 and 18 d of development. In addition, there is a significant increase in fibril diameter between 14 and 17 d of tendon development. Morphological analysis of fibrils in situ and extracted from 17–18 d tendons demonstrated extensive lateral associations of fibrils. These lateral associations (fusions) involved most of the fibril (segment) length and were not restricted to the tapered ends. This type of interaction yields longer and larger diameter fibrils.

CONCLUSIONS

Collagen assembles into fibril segments within the fibril-forming channels. Segments are D periodic structures with tapered ends which are incorporated into fibril bundles. In the developing tendon the fibril bundles are discontinuous with the segments maintained as discrete units. As development proceeds and the tendon matures, lateral fusion of fibril segments predominates. This results in longer and larger diameter fibrils. Extracellular compartments provide

a mechanism for the fibroblast to exert control over the sequential steps in fibrillogenesis. Control over the extracellular compartments would be required if, for example, the surface properties of the fibril segments must be altered to permit fusion or stabilised to prevent lateral associations. This could occur by the selected addition of a component(s) or removal at a particular stage in the hierarchy.

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