Intracellular biogenesis of collagen fibrils in 'activated fibroblasts' of tendo Achillis

AN ULTRASTRUCTURAL STUDY IN THE NEW ZEALAND RABBIT

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We have studied the formation of collagen fibrils in 'activated fibroblasts' of tendo Achillis of rabbits. The tendon was in the process of regeneration after experimental partial tenotomy. Samples were taken from the peri-incisional region and analysed by transmission electron microscopy.

Ultrastructural examination showed the presence of a 'fine dense granular substance' inside the rough endoplasmic reticulum and procollagen filaments. These come together to form collagen fibrils in the dilated vacuoles of the rough endoplasmic reticulum. The possible intra- and extracellular origin of collagen fibrils is suggested. Within the cell biosynthesis of collagen fibrils take place with the formation of collagen substance which gives rise to procollagen filaments. These make contact in parallel apposition to produce striated 'spindle-shaped bodies' which elongate by the longitudinal attachment of more procollagen filaments and form intracellular nascent collagen fibrils.

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Fibroblasts are known to synthesise collagen fibrils and mucopolysaccharides simultaneously in the ground substance in dense and in loose connective tissue.¹⁻¹⁶

Over the last 50 years there have been many studies of the extracellular formation of collagen,¹⁷⁻²¹ but its intracellular formation and excretion to the extracellular matrix have not been investigated in depth.²²⁻²⁹

Schwann³⁰ suggested that collagen fibres arose within fibroblasts and were released by splitting away from the cytoplasm, but according to Virchow³¹ fibroblasts secrete a soluble substance which is built into collagen fibres in the extracellular space. The advent of the electron microscope and autoradiography has not resolved this controversy.

Some authors^{13,32-34} defended the extracellular hypothesis of collagen synthesis by the polymerisation into collagen molecules which are excreted in the form of fibrils and also suggested the existence of an interruption of the process with intracellular modifications of procollagen molecules occurring inside the fibroblast. Others^{22,28,35-49} favoured the intracellular hypothesis and Cho and Garant,²⁵ Renteria and Ferrans⁴⁵ and Oakes⁵⁰ claimed that intracellular fibres resulted from the intracytoplasmic assembly of collagen molecules.

There have been many studies on collagen fibrillogenesis in tendons.⁵¹⁻⁶² The biogenetic pathway leading to the formation of collagen involves the rough endoplasmic reticulum (RER), the Golgi apparatus, plasma membrane transport and molecular polymerisation within the extracellular ground substance.^{13,32-34} Biochemical and ultrastructural studies also suggest that polymerisation could occur inside the fibroblast, with collagen fibrils being released by exocytosis outside the cells.⁴⁸

Procollagen molecules may be composed of different amino acids and sequences giving rise to varieties forming different tropocollagen molecules. Their synthesis depends on the mRNA exported from the nucleus and released into the cytoplasm and on the control of molecular transduction. Thus, different types of collagen are identified⁶³ the subsequent polymerisation of which gives rise to several fibrils in the ground substance of the connective tissue.⁶⁴

Collagen is the more abundant protein in the human body, representing 30% of all proteins. Biochemical, microscopic and immunohistochemical studies have identified some 19 different types (I to XIX) in the various connective tissues⁶⁵ and in recent years there have been many studies on the biochemical aspects of connective tissue fibrillogenesis.⁶⁶⁻⁸² Collagen types I, II and III have striated fibrils. Collagen type I, present in the tendon, has the thickest fibrils and has been the subject of most studies of synthesis. The genetic control of the synthesis of the differ-

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ent types of collagen has also been studied in human chromosomes.^{65,83}

Our aim in this study was to establish the different ultrastructural stages which occur in the intra- and extra-cellular biosynthesis of collagen fibrils in the regenerating tendon. These could establish the histological basis for future research on the regeneration of tendons.

Materials and Methods

We used 76 eight-month-old male New Zealand rabbits obtained from the Centre for Animal Research at the University of Alcalá de Henares, Madrid. They were kept in individual cages (30.5 × 71 × 51 cm) and were anaesthetised with an intramuscular injection of ketamine (3 mg/kg) (Rhone-Merieux, Lyon, France), xylacine (5 mg/kg) (Bayer, Barcelona, Spain) and atropine (2 mg/kg) (B. Braun Medical SA, Jaén, Spain).

We performed a partial tenotomy on tendo Achillis followed by immobilisation in plaster. Regeneration of the tendon was studied 20 and 30 days after surgery. The tendon sample was obtained from the incision which showed active regeneration. The tissue was washed in cacodylate buffer at room temperature and fixed in 2.5% glutaraldehyde-2% tannic acid in a buffered solution at pH 7.3 and 4°C for eight hours. After washing in the same buffer at 4°C for 12 hours, a second fixation in 1% osmium tetroxide at 4°C for two hours was carried out. The samples were embedded in Araldite and Spurr resins. We obtained

semithin sections which were stained with Toluidine Blue to identify the region of regeneration and ultrathin sections which were stained with lead citrate⁸⁴ or uranyl acetate⁸⁵ and observed under transmission electron microscopy.

Results

Electron-microscopic findings. The fibroblasts present at the regeneration front are known as 'activated fibroblasts' because of the extraordinary biological reaction which they undergo during tendon regeneration requiring a massive and prompt synthesis of collagen fibrils. They were elongated spindle-shaped cells with two cytoplasmic processes at each end. The interphasic nucleus, with membrane invaginations, was elongated or ovoid and was located at the centre of the fibroblast. The nucleus had a nucleolus and fine granular chromatin.

The fibroblast had an irregular morphology and some cytoplasmic regions were open to the extracellular space through vacuoles.

Intracellular findings. The few mitochondria observed were difficult to distinguish from the surrounding dense material. Dycytosomes and vesicles forming a Golgi apparatus were seldom found in these fibroblasts nor were microtubules or lysosomes. The ultrastructural cytoplasmic findings showed a large number of free ribosomes and polyribosomes usually in the form of small 'spirals' and also attached to the RER (Fig. 1) which was abundant and lay parallel to the main cell axis, with a large lumen, many

Fig. 1

Fig. 2

Figure 1 – Photomicrograph of an activated fibroblast showing ribosomes (R), procollagen filaments (thin arrows), and collagen fibrils (thick arrows) (×8000, bar 1 μm). Figure 2 – Photomicrograph of an activated fibroblast showing rough endoplasmic reticulum (RER) with dilatations and fine electron dense granular substance (*) (×25 000, bar 0.25 μm).

ribosomes and dilated at its ends (Fig. 2). The RER contained a 'fine dense granular substance' (5 to 7 nm) ('floculent electrodense material') (Fig. 2).

Many vacuoles occupied the whole of the cytoplasm at both ends of the fibroblast and contained the same fine dense granular substance and had some ribosomes attached. These were not present in the largest vacuoles. Some of the vacuoles had no boundary membranes.

Cytoplasmic areas also had a fine dense granular substance (5 to 7 nm) (Figs 1 and 3) which made contact with

Fig. 3

Photomicrograph of an activated fibroblast showing ribosomes (R), rough endoplasmic reticulum (RER), and intracellular collagen fibrils (thick arrows) ($\times 8000$, bar $0.5 \mu\text{m}$).

Fig. 5

Photomicrograph of activated fibroblast showing the Golgi apparatus, dyctiosomes (Dy), Golgi vesicles (v), procollagen filaments (thin arrows), fine electrodense granular substance (*), and elongated electrodense bodies (Eb) ($\times 6300$, bar $0.5 \mu\text{m}$).

the extracellular matrix through multiple ruptures in the plasma membrane.

In non-membrane-bound areas of the cytoplasm there were multiple untidy filaments (7 to 11 nm thick) (Fig. 2). Procollagen filaments and thin collagen fibres were present in the cytoplasmic invaginations over the nuclear infoldings and there were bundles of filaments free in the cytoplasm. Elongated non-membrane-bound spaces with 'nascent collagen fibres' (30 nm thick on average) were located in the centre of regions rich in procollagen and procollagen filaments (7 to 11 nm in diameter) which were in partial apposition to the nascent collagen fibrils (Figs 4 and 5). The last had a periodical striation (period = 67 nm).

Dense spindle-shaped bodies or fusiform bodies of different lengths with transverse striations (period = 67 nm) ('zebra bodies') composed of fibrillar non-membrane-bound aggregates were located in the RER (Figs 6 and 7). There were long non-membrane-bound intracytoplasmic

Fig. 4

Photomicrograph of an activated fibroblast showing the nucleus (N), nuclear pores (arrowheads) and bundles of procollagen filaments (thin arrows) ($\times 10\,000$, bar $0.5 \mu\text{m}$).

Fig. 6

Photomicrograph of an activated fibroblast showing vacuoles with procollagen filaments free and in bundles (thin arrows) and nascent collagen fibrils (thick arrow) with procollagen fibrils in apposition ($\times 12\,000$, bar $0.25 \mu\text{m}$).

Fig. 7

Photomicrograph of an activated fibroblast showing the nucleus (N), extracellular collagen (C), intracellular collagen fibrils (thick arrows) leaving the fibroblast (*), and electrodense bodies (Eb) (×6300, bar 1 μm).

Fig. 8

Photomicrograph of an activated fibroblast showing rough endoplasmic reticulum (RER), fine electrodense granular substance (*), electrodense bodies (Eb) and intracellular nascent collagen fibrils (thick arrow) (×8000, bar 1 μm).

spaces containing long collagen fibrils either isolated or in groups of two to four parallel fibrils (Fig. 8). Other, similar spaces open at one end, were located close to the plasma membrane of the fibroblast with some of the collagen fibres apparently leaving the cell (Figs 1 and 6).

Extracellular findings. In the extracellular region surrounding the fibroblast, there were areas containing a fine dense granular substance. Short, untidy filaments of procollagen were seen as well as long filaments some of which met in parallel apposition (Fig. 9). There were also thick (40 to 60 nm) collagen fibrils with a periodical striation of 67 nm and several intraperiodical bands.

Discussion

We have studied the ultrastructural aspects of the biosynthesis of collagen fibrils in the regenerating tendon after partial tenotomy. Fibroblasts ('activated fibroblasts') present in the regeneration front have interesting ultrastructural features which reflect the rapid synthesis of procollagen and the formation of collagen fibrils.

These activated fibroblasts are non-polarised secretory cells which release collagen from the cell surface into the extracellular matrix. Procollagen molecules and their assembly into procollagen filaments together with non-membrane-bound 'nascent collagen fibrils' were observed in the cytoplasm.

Analysis of the ultrastructural morphology of those regions inside and outside the fibroblast where the biosynthesis of collagen fibrils occurs suggests that the biosynthesis of procollagen proteins takes place in the ribosomes of the RER where they become abundant. A 'fine dense granular substance' then appears in the lumen of the RER and accumulates in the dilatations at its ends accompanied by a loss of attached ribosomes. The fine dense granular substance finally leaves the RER as smooth vacuoles that resemble the 'distended Golgi saccules' (DS) observed by Weinstock and Leblond.⁵

Fig. 9

Photomicrograph of an activated fibroblast shows the nucleus (N), fine dense granular substance (*), and intracellular collagen fibrils (thick arrow) (×3150, bar 1 μm).

Trelstad and Hayashi²² reported the presence of intracellular aggregates formed by vesicles with collagen monomers aligned in spaced longitudinal segments containing a non-striated fine dense granular substance. At the same time there is an accumulation of free ribosomes and polyribosomes outside the RER with fine dense granular substance of 5 to 7 nm.

The smooth vacuoles containing this fine substance migrate towards the periphery of the cell to excrete their contents with a partial loss of their membrane, resulting in

an irregularly-shaped fibroblast. Golgi transfer vesicles and the Golgi apparatus itself are not present in the expected amounts in these activated fibroblasts. In contrast to the observations by Cho and Gerant,²⁵ microtubules have rarely been observed in the migrating vacuoles.

The fine dense granular substance excreted by the fibroblast constitutes part of the intercellular ground substance and the collagen fibrils. Electron microscopy has shown the presence of glycosaminoglycans as granules 10 to 20 nm in size in the interstitium of the extracellular matrix. A small amount of glycosaminoglycans is associated with fibrils of larger diameter and a greater global synthesis of collagen.^{12,86} Fibrillogenesis *in situ* has been studied by Birk et al⁸⁷ and Birk, Nurminskaya and Zycband.⁸⁸

The transformation of the smooth vacuoles may occur before they reach the surface of the cell, resulting in a loss of their content inside the cytoplasm of the fibroblast which would then contain fine dense granular substance among membrane residues. Procollagen filaments 7 to 11 nm in thickness are assembled in these regions of the procollagen-containing cytoplasm outside the RER. They contact each other in parallel apposition to produce 'spindle-shaped bodies' with a periodical transverse striation of 67 nm. These striated spindle-shaped bodies may be the evidence needed to confirm the hypothesis of the intracellular biosynthesis of collagen fibrils and are in agreement with the findings of Fernandez-Madrid et al²⁴ from chicken embryo fibroblasts after treatment with colchicine and vinblastin, and of Michna²⁸ who describes striated bodies ('zebra bodies') close to membrane residues. The intracellular biosynthesis of collagen has also been studied by electron microscopy in pulmonary fibrosis in which the fibrils seem to originate as a result of the assembly of tropocollagen molecules in the cytoplasm before leaving the cell.⁸⁹

Two intracellular pathways are suggested for the biosynthesis of collagen fibrils, namely, through the lumen of the RER and secretory vesicles of the Golgi apparatus, and outside the RER and through the interstitial space of the cytoplasm where the first procollagen filaments are observed.

The intracellular synthesis of collagen is more evident in those cytoplasmic spaces limited by abundant fine dense granular substance and procollagen filaments in the cytoplasm of the activated fibroblast.

The procollagen filaments appear at the cytoplasmic spaces and contact each other in parallel apposition to constitute nascent collagen fibrils 30 nm thick. These increase their diameter (60 nm) and length by way of the attachment of procollagen filaments. A periodical transverse striation of 67 nm appears.

Using immunoelectron microscopy, Contard et al⁹⁰ showed the presence of type-I procollagen aminopropeptides (NH₂) located close to nascent collagen fibrils in cultured fibroblasts after the addition of ascorbate. Thus, long nascent collagen fibrils originate in non-membrane-bound cytoplasmic spaces. Thin, nascent collagen fibrils

with transverse striations are more often observed near the invaginations of the nucleus. The cytoplasmic spaces may contain 1, 2, 3 or more collagen fibrils which, on reaching a certain thickness and length, pass through the plasma membrane into the extracellular matrix. This would imply the existence of a process of macroexocytosis forced by the urgent need for collagen fibrils in the regenerating tendon. Collagen fibrils appear surrounded by cytoplasm either in longitudinal or in transverse sections in activated fibroblasts. Sometimes the intracellular formation of collagen fibrils does not require vacuoles or cytoplasmic spaces although they finally are located in non-membrane-bound spaces.

Lysosomes have not been observed in the intracellular synthesis of collagen fibrils in activated fibroblasts which explains the difficulty in understanding the phagocytosis and autophagocytosis theory.^{27,28,91,92} Trelstad and Hayashi²² showed that phagocytosis did not occur.

Weinstock and Leblond⁵ have demonstrated the extracellular polymerisation of collagen fibrils in activated fibroblasts. Synthesis could also occur from the fine dense granular substance excreted to the extracellular matrix by the smooth vacuoles from the RER. According to Postacchini et al⁹¹ the extracellular matrix may contain spherical dense granules interconnected by short filaments. These granules could represent initial aggregates of polysaccharide proteins which would have a role in the alignment and organisation of the collagen fibrils. We have not observed the participation of vesicles or granules in this process of collagen synthesis, possibly due to the lack of a large Golgi apparatus. Microexocytosis vesicles have not been observed in the plasma membrane of fibroblasts.

Our study suggests that nascent collagen fibrils observed in the activated fibroblast surface may have either an intracellular or an extracellular origin and that, in the case of the latter, could be formed in the longitudinal invaginations of the plasma membrane as already suggested by Trelstad and Hayashi.²² They maintained that fibroblasts may have a possible role in the process of the assembly of fibrils and indicate that a number of sites of morphogenetic regulation occur within the cell during packaging of collagen and later during the process of excretion and assembly of the fibril in the extracellular matrix. Goldberg⁹³ demonstrated high-affinity binding of soluble type-I collagen to the cell surface of the fibroblast and suggested that this may be involved in the processing of procollagen, fibrillogenesis and the regulation of collagen synthesis.

We suggest that, in activated fibroblasts, collagen synthesis occurs inside and outside the cell. Intracellular collagen synthesis is more evident in active fibroblasts due to the great demand for collagen for the regeneration of the tendon, and the formation of collagen exceeds its excretion from the fibroblast. Michna²⁸ also suggested that, on exceeding the transport capacity of the cell, synthesis of tropocollagen may occur intracellularly.

This limited capacity for transportation or translocation

Fig. 10a

RER (rough endoplasmic reticulum).
Granular substance.

Fig. 10b

RER distentions.
Granular substance.

Fig. 10c

Golgi dyctiosomes.
Golgi vesicles. →

Fig. 10d

Ribosomes.
RER.
Granular substance.
Procollagen fibrils (filaments). →

Fig. 10e

Electrondense body.
Procollagen fibrils. →

Fig. 10f

Striated electrondense body. →
Procollagen fibrils. →

Fig. 10g

Electrondense body.
Nascent collagen fibril. →

Fig. 10h

Striated nascent collagen fibril.

Fig. 10i

Collagen fibril.

Fig. 10

Stages of the ultrastructural biogenesis of intracellular collagen fibrils. Micrographs taken from the figures above as follows: a (Fig. 2), b (Fig. 3), c (Fig. 5), d (Fig. 1), e (Fig. 5), f (Fig. 4), g (Fig. 7), h (Fig. 8), i (Fig. 9).

of vesicles containing collagen molecules is supported by autoradiographic studies in fibroblasts²⁶ and electron-microscopic studies in osteoblasts.⁹⁴ Studies using the administration of colchicine^{23,61,91} also indicate that the biosynthesis of collagen could also be accelerated by illness or experimental manipulation.

We have presented the best evidence for the intracellular synthesis of collagen. When massive production of collagen exceeds the transport capacity of the plasma membrane as a consequence of blocking its secretion to the extracellular matrix, precursors assemble inside the cell.

Our findings indicate that the biosynthesis of collagen

Fig. 11a

Procollagen fibrils.

Fig. 11b

Sites of assembly between two procollagen fibrils.

Fig. 11c

Procollagen fibrils approaching by their centres.

Fig. 11d

Procollagen fibrils approaching by their ends.

Fig. 11e

Procollagen fibrils in bundle-like approaching.

Fig. 11f

Nascent collagen fibril surrounded by procollagen fibrils.

Fig. 11

Stages of the intracellular assembly of procollagen filaments. Micrographs taken from Figure 6. Thin arrows indicate assembly points of procollagen filaments.

fibrils, up to the stage of the nascent fibrils of 30 to 60 nm that are excreted into the extracellular matrix, takes place inside the activated fibroblast of the regenerating tendon after tenotomy. A fine dense granular substance is secreted and organised into procollagen filaments which make contact in parallel apposition and form spindle-shaped bodies that become striated and elongated by the longitudinal apposition of other precollagen filaments. This intracellular process of collagen synthesis is summarised in Figures 10 and 11. Synthesis may also occur outside the cell from collagen molecules excreted by the fibroblast.

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