Detection of Luse bodies, spiralled collagen, dysplastic collagen, and intracellular collagen in rheumatoid connective tissues: an electron microscopic study

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Abstract

Background—Rheumatoid arthritis is a chronic inflammatory disease leading to alterations of the extracellular matrix in tendons, ligaments, and cartilage. The structural changes of the collagenous systems in rheumatoid connective tissues are largely unknown, however.

Methods—Thirty four samples of menisci, 36 cruciate ligaments, and four tendons were taken during joint surgery in patients with rheumatoid arthritis. Eighteen menisci, 35 ligaments, and 30 tendons obtained at necropsy served as a control group. The extracellular matrix in the two groups was analysed by the combined use of transmission and scanning electron microscopy, immunohistochemistry with monoclonal antibodies recognising collagen types IV and VI, and ultramorphometry.

Results—Normal tendons and ligaments predominantly showed a unidirectional fibril arrangement. Whereas type IV collagen showed a positive staining pattern along all basement membranes, type VI collagen formed fine, filaments aligned in parallel. In patients with rheumatoid arthritis a significant reduction of the mean diameter of the collagen fibrils was found owing to the presence of thin collagenous fibrils 20–60 nm in diameter. Most of these fibrils showed considerable changes in their arrangement with irregular courses (socalled interfibrillar dysplastic collagen). Up to 410 nm thick frayed fibrils with irregular outlines (spiralled collagen) and intracellular collagen forms were found in rheumatoid tissues. In addition, atypical thick collagenous structures with 41 nm periodicity (Luse bodies) were detected in the matrix. The upregulation of type IV collagen in rheumatoid arthritis was associated with an increase in the vascular density. The expression of type VI collagen was upregulated in fibrotic zones.

Conclusions—The dramatic ultrastructural collagen changes lead to a structural and functional insufficiency of the extracellular matrix in rheumatoid connective tissues. The results suggest that collagen alterations may contribute to the development of tendon and ligament ruptures in rheumatoid arthritis.

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It is generally accepted that the extracellular matrix plays a fundamental part in modulating cellular growth and migration, maintaining tissue stability, absorbing shearing forces, and organising repair processes. These functions are guaranteed by a complex three dimensional arrangement of various collagenous and non-collagenous components. Their dynamic interplay is a decisive condition for a valid function of all connective tissues.

The collagenous fibre systems of tendons, menisci, and ligaments comprise a family of proteins with variable metabolic rates and complex functional activities. Whereas type I collagen forms a stabilising scaffold absorbing tensional forces, type III collagen fibrils seem to guarantee the pliability of tendons and ligaments and may mediate their attachment to bone cortex. Networks of type IV collagen tetramers provide the scaffold for maintaining the mechanical stability of all intraligamentous and intratendinous basement membranes. In addition, type VI collagen forms a flexible filamentous network that anchors fibrillar collagens into surrounding connective tissues.

Rheumatoid arthritis is a chronic inflammatory disease which is mainly characterised by destruction of articular cartilage, intra-synovial ligaments, and tendon tissue, which lead to extracellular matrix alterations. The underlying electron microscopic changes of the collagen fibre systems in rheumatoid tissues have not yet been exactly determined. In this study the collagen changes in this disease were analysed at the ultrastructural and immunohistochemical level.

Subjects and methods

RHEUMATOID CONNECTIVE TISSUES

Patients with definite or classic rheumatoid arthritis according to the American Rheumatism Association criteria were selected for this study. A total of 25 patients (18 women and seven men) was included in this group. Their mean age was 65–8 years and the mean duration of disease was 12–0 years. All patients...
were seropositive for rheumatoid factor and had radiological evidence of articular erosions. In total knee replacement (model according to Insall-Burstein, posterior stabilised version), 34 samples of the menisci and 36 samples of cruciate ligaments were taken. In addition, two samples of rotator cuffs and two finger flexor tendons were analysed from patients with rheumatoid arthritis.

CONTROL GROUP
Eighteen dissected menisci, 35 cruciate ligaments, and 30 supraspinatus tendons obtained at necropsy up to six hours after death served as a control group. The mean age in this group was 62.8 years. All subjects with known degenerative or inflammatory joint diseases were excluded.

TRANSMISSION ELECTRON MICROSCOPY
All specimens were analysed by transmission electron microscopy. The samples were fixed in 2% glutaraldehyde, rinsed in cacodylate buffer, and postfixed in osmium tetroxide (1%) sodium cacodylate buffer (0.2 M; pH 7.4) at 4°C for one hour. Dehydration was performed with graded ethanol (50, 70, 85, 90, 96, 100%) and the specimens were flat embedded in Spurr’s Epon (hard formula). Ultrathin sections (50–90 nm thick) were made using diamond knives. The ultracuts were collected on 2×1 mm single hole formvar coated slot grids and examined after counterstaining with uranyl acetate and Reynolds’ lead citrate for 10 minutes with an EM 10 Zeiss microscope.

ULTRAMORPHOMETRY
All meniscus ultracuts were randomised and coded before morphometric analysis. The diameter of cross sectioned collagen fibrils was measured at a final magnification of ×100 000 using a Morphomat 10 (adapted on an EM 10 microscope) with a light stylus on a tracing board. A minimum of 1000 measurements was made in each specimen. In addition, the diameters of 600 Luse bodies were measured.

The distances between the major bands of 400 collagen fibrils in each specimen and 600 Luse bodies were analysed (five measurements in each fibril). The data for the collagenous structures were analysed for statistical significance using the Wilcoxon-Mann-Whitney test.

SCANNING ELECTRON MICROSCOPY
Samples of all tissues were analysed by scanning electron microscopy. After fixation with 2% glutaraldehyde and an extended wash in phosphate buffered saline (PBS), tissues for scanning electron microscopy were osmicated for two hours, dehydrated in a graded series of acetone, and dried in liquid carbon dioxide according to the critical point method. The samples were then mounted on metal stubs with conducting paste and sputter coated with gold palladium in an evaporator (Edwards S150) before examination on the upper stage of a Stereoscan MK 250 (Cambridge) at 10 kV with a spot size of 3–10 nm.

IMMUNOHISTOCHEMISTRY
Cryostat sections (5 μm thick) of at −210°C shock frozen tissues were air-dried and fixed with cold acetone for 10 minutes. All sections were incubated with primary antibodies recognising collagen types IV and VI (table) which had been diluted in PBS (1:100), pH 7.2 for 60 minutes at 37°C in a dark humid chamber.

Streptavidine peroxidase conjugated or fluorescein isothiocyanate (FITC) conjugated antimouse IgG (Sigma) diluted 1:80 was used as the secondary antibody. After exposure for 60 minutes to this antibody at 37°C in a humid chamber, sections were washed six times for five minutes in PBS, pH 7.2, and were mounted in DABCO (0.25 g 1,4-diazabicyclo-octane (Sigma, Birmingham, United Kingdom), dissolved in 9 ml glycerol and 1 ml PBS, pH 7.2). All samples were analysed on a Leitz Orthoplan microscope using an Orthomat W camera (ultraviolet illumination with excitation wavelength of 490 nm for immunofluorescence).

Results
COLLAGEN STRUCTURE: CONTROL GROUP
Normal tendons and ligaments, obtained from subjects at necropsy, were composed of multiple, parallelly aligned collagenous fascicles. The interfascicular fibrils were mostly tightly packed and had an unidirectional arrangement (fig 1). In the centre of the human menisci,

<table>
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<td>IV</td>
<td>CC076</td>
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<td>VI</td>
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Figure 1  Longitudinal section of collagenous fibrils with unidirectional course. Note the periodic dark bands (major bands, thick arrows) on the fibrils and the brighter bands between them (minor bands, thin arrows). Transmission electron microscopy detection in a normal supraspinatus tendon.
more interlacing fibril bundles were detected than in normal tendons and ligaments. Most fibrils were aligned in parallel (fig 2) on the surface of the menisci.

**Figure 2** Thin collagenous fibrils (thin arrows) on the intact surface of a normal human meniscus. The fibrils were predominantly arranged in parallel. Note the fine network of fibrin that covers the fibrils (thick arrows) (scanning electron microscopy).

**Collagen Structure: Patients with Rheumatoid Arthritis**

**Collagen Dysplasia**

In rheumatoid connective tissues the collagen fibre bundles on the surface of the tissues were altered, fragmented or ruptured (fig 3). At higher magnifications, many thin irregularly shaped fibrils were detected (fig 4). In deeper zones, the collagenous fibril systems also showed severe alterations. In transmission electron microscopy there was often a complete loss of the regular collagen architecture in rheumatoid arthritis with dramatic ultrastructural collagen anomalies. This collagen disorganisation was often also present in histologically unchanged regions. Especially in matrix vesicle rich areas near infiltrating synoviocytes, severe changes of the interfibrillar collagen arrangement were found (fig 5). The packing of fibrils within a fibre was highly disorganised. Single fibrils showed atypical unparallel courses or followed an angular or zigzag-like course. These thin, so-called 'interfibrillar dysplastic' collagen fibrils were found in all rheumatoid samples. Their diameter was usually between 30 and 60 nm.

**Spiralled collagen**

In most rheumatoid tissues individual fibrils resembled the so-called 'collagen flowers'. These fibrils had a typical stellate appearance with a central thick core, thin peripheral elements, and small connecting bridges (fig 6). Their diameter was up to 410 nm. In cross sections these fibrils had a frayed or ravelled appearance with great variability in width and shape, whereas in longitudinal sections a helicoidal arrangement of the constituent fibrils and filaments was observed. In contrast with dysplastic fibrils, frayed fibrils occurred in vesicle poor areas.

**Luse bodies**

Many fibroblasts in patients with rheumatoid arthritis secreted flocculent, unstructured material in the extracellular space (fig 7). After secretion in the matrix these atypical thick collagenous structures (so-called 'Luse bodies') became more structured and showed a longitudinal arrangement (fig 8). Although most of them showed no periodic banding pattern, others had a reduced periodicity between their major bands (fig 9).

**Intracellular collagen**

In all rheumatoid tissue samples, an increase in the number of fibroblasts, monocytes, and mononuclear macrophages was seen. In the cytoplasm of the fibroblasts and the macrophages, tubular and vesicular structures containing membrane-bound collagen fibrils were often seen (fig 10). There was a narrow space between this membrane and the collagen fibrils often containing an electron dense granular material. Their diameter was usually about 50 nm. These phagosomes with
intracellular collagen fused with primary lysosomes and then formed heterolysosomes. In these secondary lysosomes the collagen fibrils often showed irregular outlines, became homogeneous, or appeared unbounded.

COLLAGEN ULTRAMORPHOMETRY: CONTROL GROUP
There was a characteristic unimodal distribution of the diameters of the collagenous fibrils in the menisci. The fibril diameters ranged from 20 to 190 nm (mean (SD) 68.1 (10.2) nm). All collagenous fibrils showed a periodic banding pattern. The distance between their major bands (fig 9) ranged between 50 and 66 nm (mean (SD) 57.1 (6.1) nm).

COLLAGEN ULTRAMORPHOMETRY: PATIENTS WITH RHEUMATOID ARTHRITIS
Although the range of the fibril diameters was increased in menisci from patients with rheumatoid arthritis (mean (SD) 20–410 (32.6) nm), the mean fibril diameter in rheumatoid arthritis was significantly (p<0.001) reduced (58.2 nm). The morphometrically determined distance between the major bands of the collagenous fibrils was significantly (p<0.05) reduced compared with the control group (fig 9). The periodicity of the collagen fibrils in patients with rheumatoid arthritis ranged from 44 to 62 nm (mean (SD) 51.5 (6.7) nm).

The Luse bodies in rheumatoid tissues showed a more pronounced and significant (p<0.01) reduction of the distance between their major bands (mean value 41 nm) ranging between 26 and 62 nm. Luse bodies were significantly (p<0.001) thicker than other collagenous structures in rheumatoid menisci.

COLLAGEN TYPES: CONTROL GROUP
Type IV collagen was found in all vascular basement membranes of tendons, ligaments, and menisci. Therefore, arteries and veins in the ligaments were clearly delineated by the
antibody to type IV collagen. The binding pattern was restricted to these vascularised areas, however, whereas a staining of cells or intercellular matrix could not be convincingly shown.

Immunostaining with the antibody against type VI collagen showed filamentous structures in the extracellular space of all tissues, which bound regions between large collagenous bundles. Most type VI collagen filaments were arranged in parallel.

COLLAGEN TYPES: PATIENTS WITH RHEUMATOID ARTHRITIS
The expression of type IV collagen was substantially increased in patients with rheumatoid arthritis. This finding was associated with a hypervascularisation of rheumatoid connective tissues. Even in normally avascular zones of the menisci, vessels and type IV collagen were detected in rheumatoid arthritis (fig 11). There was a considerable increase in the amount of type VI collagen structures in fibrotic zones (fig 12). In other areas the content of type VI filaments was unchanged or even reduced compared with the control group. Some filaments in rheumatoid arthritis were more wavy or ruptured. No atypical thick structures acceptable as ‘Luse bodies’ were delineated by the antibody.

Discussion
The synovial membrane in rheumatoid arthritis is characterised by cellular infiltrations, increased vascularity, lining layer hyperplasia, and interstitial fibrosis. This is accompanied by an increase in expression of extracellular
Collagen ultrastructure in rheumatoid arthritis

matrix components, such as fibronectin, tenascin/f1, and several collagen types, leading to their enhanced deposition in the matrix. On the ultrastructural level, many thin collagen fibrils were detected causing a reduction in the average fibril diameter in rheumatoid tissues. This may be explained by a synthesis of young fibrils in rheumatoid arthritis. A fragmentation or splitting of fibrils in subfibrillar elements may also contribute to this finding.

Whereas most fibrils in rheumatoid arthritis were thinner than in the control group, other fibrils were atypically thick. These flower-like fibrils are also termed ‘spiralled collagen’, ‘composite fibrils’, ‘hyperfibrils’, or ‘frayed fibrils’. Their occurrence is thought to be a degenerative phenomenon in altered lumbar spine ligaments, inherited collagen disorders (for example, Ehlers-Danlos syndrome), and in fibrotic tissues undergoing remodelling. In rheumatoid arthritis hyerfibrils represent the highest degree of collagen polymerisation as these fibrils are probably derived by a falling together of some minor fibrils. It has been suggested that these fibrils reflect breakdown states of collagen fibrils in vivo and that their origin is due to confrontation with degradative, proteolytic enzymes. In contrast with dysplastic fibrils, however, hyperfibrils in rheumatoid arthritis were mostly found in vesicle poor, non-inflamed regions. It may therefore be speculated that hypoxia may also be an important condition for their development in this disease.

One of the most impressing features of collagen alterations in rheumatoid connective tissue consisted of the presence of changes in the interfibrillar collagen architecture. The fibrils lost their typical parallel unidirectional arrangement and became interfibrillar dysplastic. Interfibrillar dysplastic fibrils were first described in degenerative vessel diseases. They may also occur in tendons after treatment with anabolic steroids, in anterior cruciate ligament ruptures, and in chronic pancreatitis. Their origin is probably related to a release of degradative enzymes from calcifying matrix vesicles (so-called Bonucci concept) released by infiltrating synovial fibroblasts, and by macrophages.

The appearance of dysplastic fibrils may therefore be due to a collagen confrontation with enzymes, such as neutral matrix metalloproteinases, secreted by infiltrating synovial cells. These aggressive enzymes can influence the critical balance between proteolysis and proteinase inhibition in rheumatoid arthritis and perhaps may lead to collagen alterations and the development of dysplastic collagenous fibrils in this disease. The detection of dysplastic fibrils in rheumatoid arthritis is an important finding, however, because it was suggested that these fibrils may not withstand normal tensile forces, probably due to a reduction of their intrafibrillar covalent cross links and their structural disorganisation. Interfibrillar dysplastic fibrils therefore lead to a structural and functional insufficiency of the rheumatoid matrix.

Luse bodies (or zebra bodies) were first described by Luse in 1960 and 1963. Although Cravioto and Lockwood suggested that they were fibrous long spacing collagen equivalents, Imura et al. presumed that they were segment long spacing collagen aggregates. These terms had been used to designate collagen fibrils where the periodicity is markedly greater than the 64 nm periodicity of the common collagen fibril. It is thought that in fibrous long spacing collagen the 260 nm long tropocollagen molecules come together side by side so that the length of the period is approximately the same as the length of the tropocollagen molecule. The distance between the major bands of the Luse bodies in rheumatoid connective tissues was, however, reduced and not increased compared with control tissues. Therefore they probably do not represent fibrous long spacing collagen fibrils. It seems more likely that they are degenerative collagen structures that develop after the cellular secretion of flocculent, unstructured material followed by a pathological extracellular maturity process. There was no clear evidence from the immunohistochemical studies that type VI collagen is involved in the formation of Luse bodies.

Intracellular collagen has been previously described in torn menisci, in mouse decidual cells, in the postpartum uterus, and in periodontal ligaments. It has been interpreted as phagocytosis of collagen with intracellular fibril degradation, or as a result of an enhanced collagen synthesis with intracellular fibril formation. In rheumatoid connective tissues, intracellular collagen was found in phagosomes of macrophages and fibroblasts. This finding is supported by previous ultrastructural studies. The fusion of collagen containing phagosomes with primary lysosomes, the loss of fibril banding, and the homogenisation of the fibrils in the lysosomes suggest an intracellular degradation of collagen in rheumatoid patients. It is suggested that phagocytosis of collagen may be a mechanism of matrix remodelling in rheumatoid connective tissues.
The ultrastructural changes of the collagenous fibril systems lead to dramatic structural and functional alterations of connective tissues in rheumatoid arthritis. The normally highly organised tendons and ligaments are therefore not more structured to resist normal tensional stresses and to absorb shearing forces. It is suggested that this finding contributes to a functional insufficiency of these tissues in rheumatoid arthritis and explains the development of tendon and ligament ruptures in this disease.

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