Cellular ultrastructure of the ruptured anterior cruciate ligament

A transmission electron microscopic and immunohistochemical study in 55 cases

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To evaluate the cellular ultrastructure following injury, we examined the anterior cruciate ligaments in 55 patients with complete tears in different phases after the injury and compared them to a control group of 39 cadaver knees. Samples were analyzed by electron microscopy, immunofluorescence, and ultramorphometry. After an invasion of inflammatory cells into the stumps of the ruptured ligaments, a marked proliferation of fibroblasts was found at the end of Phase 1 (2–3 days after the ligament injury), that was even more pronounced at the beginning of Phase II (4–17 days). These cells were initially highly metabolically active and secreted Type III collagen precursors. In Phase III (4–45 days), fibroblast degeneration occurred with increasing frequency. Furthermore, some fibroblasts showed signs of cell death. Our findings suggest that the structural alterations of the intraligamentous fibroblasts diminish their function and, consecutively, disorganization of the developing repair tissue occurs. This mechanism might contribute to the poor healing potential of the ruptured anterior cruciate ligament.

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During the healing process of ruptured extraarticular ligaments, 4 different phases occur to form a ligamentous repair tissue (Andriacchi et al. 1988, Arnoczky 1991): Phase I, within the first 72 hours after injury, is characterized by an acute inflammatory reaction; Phase II, which occurs over the next 6 weeks, is associated with a marked proliferation of both cellular and extracellular components; Phase III, ligament remodeling which usually takes several weeks to several months; and Phase IV, final remodeling with maturation of the scar matrix which can take several months to years.

Differences in biological healing responses between the medial collateral ligament and the anterior cruciate ligament following injury are well accepted. Whereas the extrasynovial collateral ligament heals rapidly following acute disruption, the subsynovial anterior cruciate ligament frequently shows no effective healing response, even in situations where repair is attempted (Woo et al. 1990, Hefti et al. 1991, Hefti 1992, Johnson et al. 1992). While exploring of these differences in healing, recent experiments have identified several factors that appear to be involved in this phenomenon, including the complex anatomy of the anterior cruciate ligament and its critical vascular supply (Reiman and Jackson 1987, Arnoczky 1991, Lyon et al. 1991, Neurath and Stofft 1993). In an attempt to further analyze these factors behind poor healing capacity, several groups (Ross et al. 1990, Nagineni et al. 1992) used cell culture studies of rabbit fibroblasts. They found a slower rate of proliferation and a lower secretion of Type III collagen of anterior cruciate ligament cells compared to those of the medial collateral ligament under normal culture conditions suggesting differences in their intrinsic capacity. However, with the cells of the human anterior cruciate ligament, the experimental approaches have given less definite results.

We characterized the spontaneous healing process of the completely ruptured human anterior cruciate ligament and analyzed structure and possible function of its cellular systems during different phases after the injury.

Patients and methods

Anterior cruciate ligament rupture

The study group consisted of 34 men and 21 women with a mean age of 30 (15–59) years. All had a complete tear of an anterior cruciate ligament. Patients with known metabolic disorders were excluded. The interval between injury and surgery of the ligament ranged from 2–56 days. The time interval served to classify the ruptured ligaments into 3 different phases after the injury: 21 anterior cruciate ligaments were in Phase I, 29 in Phase II, and 5 in Phase III. The biopsies were taken both directly and 1–3 cm aside from the rupture zone.

Control group

Anterior cruciate ligaments from 39 fresh cadavers with no history of knee injuries or metabolic diseases were used for comparison. The average age of the controls was 35 (15–60) years. To minimize autolytic changes, all ligaments were taken within 7 hours after death.

Transmission electron microscopy

Immediately after removal, each sample was placed in Karnovsky's fixative for at least 12 h at 4 °C and was then sliced longitudinally into specimens of approximately 1 × 2 × 2 mm. The pieces were then postfixed in 1% osmium tetroxide buffered with 0.2 M sodium cacodylate buffer (pH 7.4) for 1 h. Thereafter, the tissues were dehydrated serially in graded ethyl alcohol and embedded (via propylene oxide) in Spurr's Epon (hard formula). 50–90-nm ultra-thin sections were made, placed on grids and stained with uranyl acetate and aqueous lead citrate. All sections were viewed with a Zeiss EM 10 transmission electron microscope.

Ultramorphometry

For detection of pathological changes of the ligamentous fibroblasts, 1 mm^2 of each specimen was analyzed with a semiautomatic Morphomat 10-image analysis system (adapted on an EM 10) on a tracing board. The ultrastructural data of the fibroblasts between the 4 groups were compared using multivariance analysis.

Immunohistochemistry

For immunolocalization of Type III procollagen, monoclonal antibodies (mouse anti-human IgG, Dianova, Hamburg) were used. Unfixed samples of the ligaments were quick-frozen in liquid nitrogen at −196 °C. 5–10-µm cryosections were made using a Frigocut 2800, air-dried, fixed with cold acetone for 10 min, and incubated in antibody-containing fluid (diluted 1:150 in phosphate-buffered saline) overnight in a dark humid chamber. Following a wash in phosphate-buffered saline over 2 h, secondary antimouse antibodies (fluorescein-isothiocyanate conjugated antimouse IgG, obtained from Sigma, Munich), diluted 1:100 in phosphate-buffered saline and supplemented with 1% human serum, were applied for an additional 3 h at 37 °C. All samples were then analyzed under UV-illumination (excitation wavelength 490 nm).

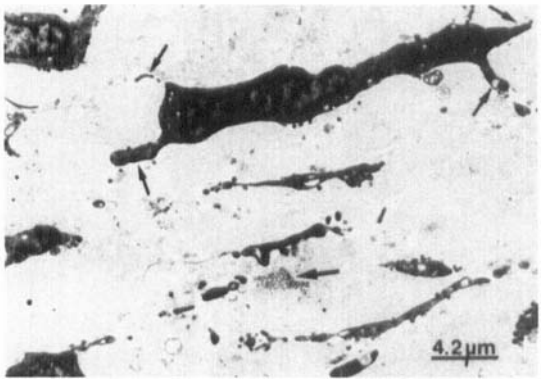

Figure 1. A fibroblast with several cytoplasmic prolongations (thin arrows) and longitudinal shape in the anterior cruciate ligament. An oxytalan fiber is also visible (thick arrow). Control group, ×3500.

Results

Control group

In the normal anterior cruciate ligament, the majority of cells in the outer zones of the ligament demonstrated a longitudinal shape with several cytoplasmic prolongations (Figure 1). However, cells with oval shape were more frequent in central areas of the ligament. Rough endoplasmic reticulum and Golgi vesicles in the cytoplasm of these cells were only sparsely developed. Strict evaluation of their cellular ultrastructure revealed one or more pathologic changes in 5 percent of the studied cells (Table 1).

Ruptured ligaments, Phase I

Within the first 3 days after the injury, a marked inflammatory reaction was observed in the stump of the anterior cruciate ligament. Erythrocytes (Figure 2), lymphocytes, and mononuclear macrophages were common. Surprisingly, macrophages were found also 2–3 cm away from the rupture zone (Figure 3). In the extracellular matrix, fibrinous exudate and cell debris were observed predominantly near the rupture area.

At the end of this phase, a marked proliferation of fibroblasts occurred in the ruptured ligaments (Figure 4). Those cells showed an abundant rough endoplas-

Table 1. Frequency of anterior cruciate ligament fibroblasts with pathologic ultrastructure

	Control	Phase			
		I	II		III
Number of specimens	23	21	18	11	5
Days after injury		1–3	4–24	25–45	>45
Vesiculation of cytoplasm[a]	4	23	29	38	28
Rough endoplasmic reticulum					
Degranulation[a]	0.4	5	8	19	11
Dilated cisternae[a]	1	9	25	35	20
Lipid droplets in the cells[a]	0.2	1	8	9	7
Increased amount of filaments[a]	1	7	29	45	30
Percent of fibroblasts with pathologic findings	5	26	40	54	38

[a] All values in percent. Wilk's lamdba < 0.01

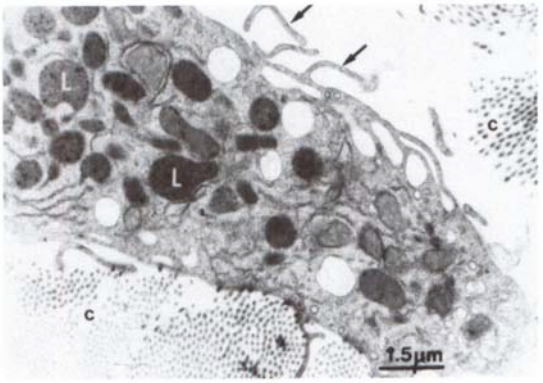

Figure 3. Mononuclear macrophage at a distance of 2 cm from the rupture zone. Note its typical filopodia (arrows) and abundance of lysosomes (L). The collagenous fibrils (c) are still well preserved. Phase I after the ligament injury, ×5500.

Figure 2. In Phase I after the ligament injury, erythrocytes (E), cell debris (arrows), fibrin (F), and degenerated cytoplasmic prolongations (cp) were common near the rupture zone, ×12500.

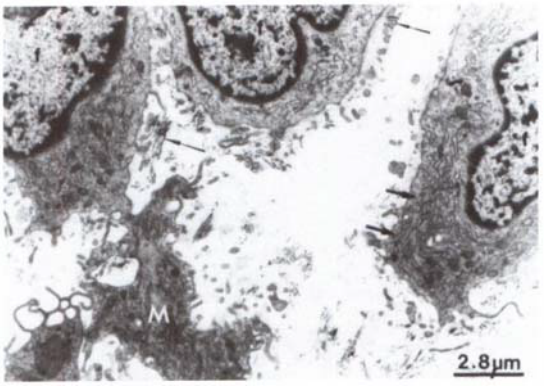

Figure 4. In addition to the presence of macrophages (M), a marked proliferation of fibroblasts (f) was found at the end of Phase I after the ligament injury. The fibroblasts showed a well developed rough endoplasmic reticulum (thick arrows). Thin collagenous fibrils were seen in pericellular areas (thin arrows) suggesting a collagen synthesis of those cells, ×5500.

mic reticulum and, occasionally, several Golgi vesicles or 2 nucleoli. Although there was no striking difference between the ligaments of younger and older patients, the proliferation of fibroblasts in the anterior cruciate ligament of older patients usually was less pronounced.

A markedly enhanced expression of Type III procollagen in pericellular areas began on the third day (Figure 5).

Phase II

In the beginning of this phase, the proliferation of fibroblasts in the stumps of the ruptured anterior cruciate ligaments was even more pronounced than in Phase I. However, their cellular ultrastructure was increasingly distorted. Many fibroblasts near the rupture zone showed severe degenerative alterations, including dilated cisternae of the rough endoplasmic reticulum and intracytoplasmic lipid droplets. In addition, the cells contained many intermediate filaments about 9 nm in diameter. Another finding was degranulation of the ribosomes of the rough endoplasmic reticulum, leading to irregular ribosomal accumulations (Figure 6).

Furthermore, more and more fibroblasts became necrotic with increasing time after the injury. In some of these cells, the small nucleus revealed true inclusions or pseudoinclusions (Figure 7). The cytoplasm showed many vesicles that sometimes contained chromatin. Finally, the nucleus became homogeneous, the plasma membrane dissolved, and karyolysis occurred. The collagen fibrils in the vesicle-rich areas frequently showed a stellate appearance, so-called collagen hyperfibrils.

The ultrastructural changes were found not only at the rupture zone but also, with decreasing frequency,

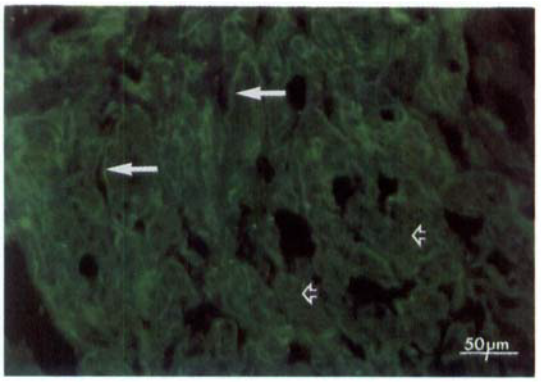

Figure 5. Type III procollagen (long arrows) expression in pericellular areas. Note the large number of cells within the ligament (short arrows). Phase I after the ligament injury. Indirect immunofluorescence, ×400

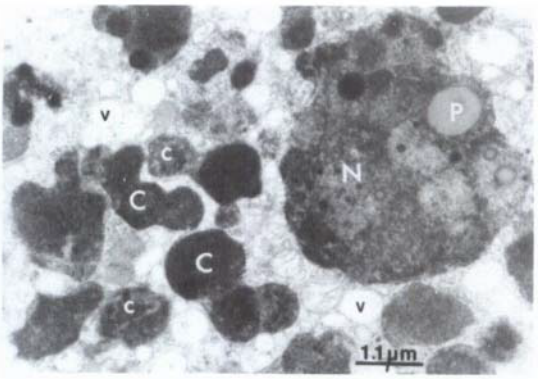

Figure 7. A degenerative anterior cruciate ligament fibroblast in Phase II after the ligament injury. Its remaining nuclear fragment (N) showed severe alterations and numerous inclusions (P). Some of its cytoplasmic lysosomes (C) contained chromatin (c) indicating an irreversible cell damage. Several cytoplasmic vacuoles are also visible (V), ×13600.

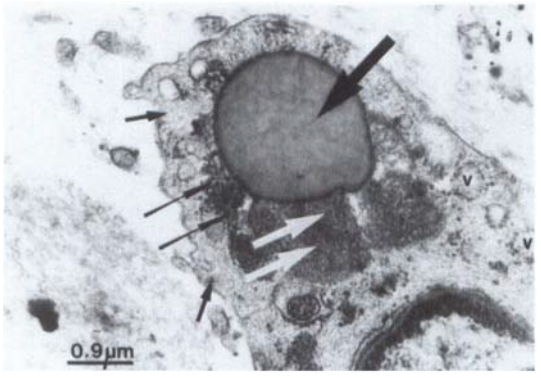

Figure 6. Severe degenerative alterations of a fibroblast in Phase II after the ligament injury. Many intermediate filaments (short arrows), vesiculated cisternae of rough endoplasmic reticulum (v), pathological ribosomal accumulations (long thin arrows), degraded ribosomes (white arrows), and a lipid droplet (thick arrow) were found in the cytoplasm, ×17000.

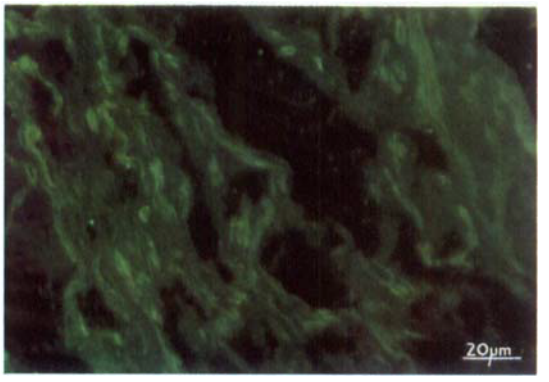

Figure 8. In the beginning of Phase II after the ligament injury, a high Type III procollagen expression was observed in the stump of the ruptured anterior cruciate ligament, ×1000.

several centimeters away from the rupture area. Surprisingly, there were only few myofibroblasts in the ligament stumps during Phase II.

The expression of Type III procollagen showed a peak between the fourth and seventh days after injury (Figure 8) and then steadily decreased.

Phase III

In all the 5 stumps of the cruciate ligament taken at this phase, the number of macrophages and fibroblasts was reduced compared to Phase II. The fibroblasts contained only a sparsely developed endoplasmic reticulum. However, there was a higher variability in the zone-to-zone cellular ultrastructure varying more from patient to patient in comparison with Phase II. Although there were no clear qualitative differences in alterations of the ligament fibroblasts, the overall impression was a slight reduction in the number of altered fibroblasts compared with Phase II, but these differences were not significant. However, the repair tissue in the stumps of the anterior cruciate ligament never approached normal ligament characteristics and no adequate tissue remodeling occurred. Neither the gender nor the age of the patients influenced this finding.

Discussion

Within the first 3 days after the complete rupture, many inflammatory cells migrated into the stump of the anterior cruciate ligament. Interestingly, mononuclear macrophages also were seen far away from the rupture area. Their functions probably include phago-

cytosis and degradation of cellular and extracellular components by hydrolytic enzymes. Furthermore, it has been speculated that these cells secrete angiogenetic factors that might contribute to the marked vascular response observed in some ruptured anterior cruciate ligaments (Andriacchi et al. 1988, Arnoczky 1991).

It is generally accepted that each healing phase of collagenous tissues is critically dependent on fibroblast structure and function (Komuro 1990, Regan et al. 1991). At the end of Phase I after injury, a marked proliferation of fibroblasts was observed in the stumps of the ruptured anterior cruciate ligament and the phenomenon was even more pronounced in Phase II. Fibronectin, that is remarkably up-regulated in ruptured cruciate ligaments (Neurath and Printz 1992), may modulate fibroblast chemotaxis and functional activity (Gelberman et al. 1991), and in this way collagen neoformation.

In our study, the abundance of fibroblast endoplasmic reticulum and the high expression of Type III procollagen in pericellular areas indicated that, initially, fibroblasts were metabolically highly active and secreted collagen precursors in the extracellular matrix. We found that a high Type III procollagen secretion started on Day 3. Interestingly, we found in subsequent studies with reverse transcriptase-polymerase chain reaction (RT-PCR) that a marked synthesis of Type III collagen messenger RNA in the fibroblasts of the anterior cruciate ligament occurred even within the first 24 h.

In Phase II, severe ultrastructural alterations of the fibroblasts were detected with increasing frequency, including ribosomal accumulations. These pathologic changes are known to be a useful indicator of depressed or arrested cellular protein synthesis (Ghadially 1988). Together with the decreasing Type III procollagen expression during Phase II, our data strongly suggest that the fibroblasts in the stumps of the ruptured anterior cruciate ligament have only a limited collagen synthesis capacity and thus a limited capacity to support the healing process during this important phase. Finally, several of those cells became necrotic even at a distance of 2 or 3 cm from the rupture zone. In addition, no relevant amount of myofibroblasts was found in the stumps of the anterior cruciate ligament, although these cells are thought to be important in normal healing processes (Hasegawa et al. 1990, Komuro 1990).

Although some of the observed degenerative ultrastructural fibroblast alterations resembled those described in ligaments and synovial tissue of immobilized rat and rabbit knee joints (Ghadially 1988, Newton et al. 1990, Padgett and Dahners 1992), the severity and frequency of the lesions suggested that fibroblast alterations in the ruptured ligaments were not simply induced by preoperative immobilization. Other factors that might have been important in fibroblast degeneration are the poor nutrition of the anterior cruciate ligament, possibly inducing cell hypoxia, and catabolic enzymes that may alter these cells.

The location of the anterior cruciate ligament has been referred to as the hostile environment of the synovial joint space. Evidence supporting this model has been obtained from recent studies that showed markedly elevated concentrations of aggressive enzymes (collagenase/matrix metalloproteinase 1) in the synovial fluid and injured anterior cruciate ligament itself following trauma (Amiel et al. 1990, Walakovits et al. 1992). Fibroblast proliferation in the completely ruptured anterior cruciate ligament could be reduced when exposed to synovial fluid that contains aggressive enzymes. On the other hand, fibroblasts themselves can produce collagenase (Aggeler et al. 1984, Konttinen et al. 1991) which, in injuries, contributes to the degradation of their matrix. Existence of collagen hyperfibrils around necrotic fibroblasts in stumps of the ruptured anterior cruciate ligament provides direct evidence for collagen degradation, since the development of those fibrils is due to collagen interaction with proteases (Emonard et al. 1991). The alterations of fibroblasts probably cause a diminished functional activity of those cells and may explain the low Type I procollagen synthesis in ruptured anterior cruciate ligaments (Wiig et al. 1991). The limited capacity of the intraligamentous fibroblasts to support the healing process might contribute to the matrix disorganization (Neurath and Stofft 1992) and to the poor healing potential of the ruptured anterior cruciate ligament.

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