FIBROBLASTIC HEALING OF GRADE II LIGAMENT INJURIES

HISTOLOGICAL AND MECHANICAL STUDIES IN THE SHEEP

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A method is described which produces an isolated Grade II injury in the medial collateral ligament of the sheep's knee. The untreated injury was followed in 32 animals for up to six weeks. Histology revealed progressive healing of the lesions by a cellular response mediated by fibrocytes rather than by the classically described inflammatory response. Mechanical testing showed that the tensile strength of the ligament was initially reduced to 13% of normal but that strength and compliance were regained after six weeks.

It has been assumed that ligament repair is effected by scar tissue which arises from an initial inflammatory reaction mediated by cells extrinsic to the ligament (Frank et al. 1985). This concept of ligament repair appears to have been reached as a result of three types of study:

First, direct transference of results obtained from the more extensively investigated process of repair in tendons; a recent book on ligamentous injuries bases its chapter on repair entirely on findings in tendons (Jenkins 1985). However it has been shown that tendons are histologically distinguishable from ligaments (Amiel et al. 1984), and since tendons have a more vascular sheath than ligaments, direct comparison of repair between the two tissues cannot be justified.

Secondly, concepts of ligament repair have been based mainly on animal models where the ligament has been either completely or partially sectioned by cutting at a single site (Horwitz 1939; Clayton and Weir 1959; O'Donoghue et al. 1961; Kappakas et al. 1978; Arnoczky, Rubin and Marshall 1979; Cabaud, Rodkey and Feagin 1979; Fukubayashi et al. 1980). This type of model is of limited clinical relevance as in most patients the whole ligament is excessively stretched and damaged before one part of it ruptures. This deficiency was acknowledged by some of the authors (Clayton and Weir 1959; O'Donoghue et al. 1961).

Thirdly, from attempts at employing a more physiological mechanism of injury in animals which resulted in variable lesions ranging from no tissue damage to complete rupture (Miltner and Hu 1933; Jack 1950).

In clinical practice a Grade II ligament injury is more commonly seen than the complete rupture of Grade III. A Grade II lesion arises because the magnitude of the load is such that it exceeds the peak tensile strength of the ligament yet does not reach its breaking strength (Kennedy et al. 1976). Thus the ligament is severely weakened but remains physically intact and retains some mechanical strength, as clinically demonstrated by a firm end-point on stressing.

There have been no successful attempts at producing a consistent and reproducible animal model of a Grade II ligament injury (Miltner and Hu 1933; Miltner, Hu and Fang 1937; Jack 1948, 1950). It is, however, everyday clinical experience that such ligament injuries do undergo repair. This process warrants investigation but, because these injuries are rarely operated upon, biopsy specimens cannot be obtained from human tissue.

We have studied the repair of Grade II ligament injuries in an animal model, producing such injuries in the medial collateral ligament (MCL) of the knee joint of the sheep, and studying the natural history of the untreated lesion both histologically and mechanically.

MATERIALS AND METHODS

Thirty-two skeletally mature (5 to 6-year-old) cross-bred ewes were used. The MCL of the left knee was subjected to a standard injury, with the right uninjured knee serving as a control. Eight animals were killed immediately after injury, and eight at each of one, three and six weeks later. Four animals at each time interval were used for histological examination and four for mechanical testing.

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Fig. 1

Diagram to show the position of the three pins. There is an 8 mm femoral pin, an 8 mm upper tibial pin and a lower 4 mm Steinmann pin.

Surgical procedure

Placement of pins (Fig. 1). Under halothane and nitrous oxide anaesthesia both hind legs were shorn, the exposed skin degreased and a mild antiseptic applied. Under image intensifier control, an 8 mm diameter, unthreaded, stainless-steel pin was drilled into the medial aspect of the femur in a mediolateral direction, at a point mid-way between the anterior and posterior borders of the medial femoral condyle and just proximal to the medial epicondyle. Precise positioning of this pin was critical as it had to avoid the insertion of the MCL yet had to penetrate sufficient bone to provide effective mechanical purchase. The pin was advanced through the lateral soft tissue and skin to project equally from each side of the leg.

A 2 cm mid-line incision was then made through the patellar tendon to the tibial tuberosity and a second 8 mm diameter pin was drilled through the tuberosity in an anteroposterior direction. A slight medial and distal angulation ensured the maximum hold in bone. This pin passed just through the posterior cortex but not the soft tissues.

Through a small stab incision on the medial aspect of the tibia, approximately 5 cm distal to the insertion of the MCL, a 4 mm diameter Steinmann pin was drilled through the tibial shaft approximately parallel to the 8 mm diameter femoral pin. This 4 mm pin was subsequently used in the measurement of varus/valgus laxity.

Measurement of joint laxity. Joint laxities were measured before and after injury as well as immediately post mortem. To measure *medial and lateral rotation* the knee

was held in 45° of flexion to relax the posterior capsule. A standard torque force was applied to the joint by attaching a spring balance to the 8 mm tibial pin, at 5 cm from the skin. A steady force of 5.5 kg in medial, then lateral, rotation was applied at right angles to the tibial pin and rotation of the tibial pin relative to the femoral pin was measured by protractor.

Varus and valgus laxities were measured at 20° of flexion to relax the posterolateral and posteromedial capsule. The hook of the spring balance was placed against the skin at the base of the 4 mm tibial pin. A rigid flat card bridging the 4 mm tibial and 8 mm femoral pins was attached to the 4 mm pin and a line then drawn on the card along the femoral pin. A steady load of 5.5 kg was then applied through the spring balance parallel to the tibia and a new line drawn on the card along the femoral pin. The angle between the two lines was then measured. Both valgus and varus laxities were measured.

Injury. Each sheep was positioned on its side so that its left hind limb was over the edge of the table. The now vertical femoral pin was held very firmly to prevent movement while the other experimenter held the leg with one hand so that the knee was maximally flexed and with the other hand grasped the 8 mm tibial pin. Through this pin the tibia was laterally rotated by manual force until a characteristic sound caused by soft-tissue tearing was heard from the region of the MCL. After post-injury measurements of laxity, the three pins were removed and the wounds closed. For the control right hind limb the same procedures of pin insertion and measurement were followed but the ligament was not injured.

The animals were maintained overnight in a sling. They were then transferred to a large pen for one week, by which time almost all had ceased limping. They were then released into open fields.

Post-mortem examinations. Immediately post mortem the three pins were reinserted into the same holes and measurements of joint laxity were repeated. Specimens were then excised for either mechanical testing or histology.

Mechanical testing. The ultimate tensile strength and compliance of both the left and right MCL were determined. Each knee was excised with 7 cm of bone on each side of the joint. Parallel 8 mm diameter holes were drilled mediolaterally, one through the femur at the junction of the shaft and condyles and the other 2 cm distal to the tibial attachment of the MCL. Pins passed through these holes were used to secure the specimen to the clamps of an Instron 1193 materials testing machine. The undissected specimens were kept moist with normal saline and all testing was carried out within 2 hours of death. All the soft tissues except the MCL and its meniscal attachments were then resected. The ultimate tensile strength of the femur-MCL-tibia complex was then measured at room temperature at a speed of 500 mm/min.

Histology. The whole MCL, including its insertions into

the bone, was excised and cut into three sections: tibial insertion, femoral insertion and a central section with the meniscus still attached. Sections were prepared and stained with either haematoxylin and eosin or Mallory's trichrome.

RESULTS

There were no wound infections. The means and standard errors of the results were calculated for each parameter in each group of knees. Values were obtained for statistical significance between the results at the different time intervals, using paired, two-tail t-tests, except for those for ultimate tensile strength and compliance where unpaired, two-tail t-tests were employed. The results recorded before injury were compared statistically with the results at the various time intervals after injury.

Joint laxity (Fig. 2). Before injury there were no significant differences between the laxities of the right and left knees.

Lateral rotational laxity rose significantly after injury ($p < 0.01$) but returned to normal by one week.

Medial rotational laxity showed no significant changes.

Varus laxity did not change significantly.

Valgus laxity. In all cases there was a firm end-point to stressing the MCL. A highly significant ($p < 0.001$) increase in laxity was present immediately after injury and this remained significant at one week ($p < 0.02$) and at three weeks ($p < 0.01$), but by six weeks it was not significantly different from the pre-trauma values.

Mechanical testing. In almost all specimens the bone-ligament-bone preparation failed under tensile loading by rupture of the mid-portion of the ligament. In five uninjured animals the ultimate tensile strength of the right and left MCL was compared and found to have no statistically significant difference ($p = 0.9$). The ultimate tensile strength varied considerably between animals (range 48 kg to 109 kg) and therefore the ultimate tensile strength of an injured ligament was expressed as a percentage of that of the contralateral, uninjured ligament.

Results are presented in Figure 3. Immediately after injury the strength of the ligaments was reduced, on average, to 13.3% of that of the controls ($p < 0.001$). By one week this had increased slightly to 14.9%. At three weeks the mean strength was 39.1%; this being significantly different from pre-injury ($p < 0.01$), immediate

Fig. 2

Diagrams to show changes in joint laxity in four parameters. For each one the mean $\pm 2 \times$ SE is given in relation to pre-trauma levels. An initially significant increase ($p < 0.01$) in lateral rotation (upper left) has returned to normal by one week. There are no significant changes at any time in medial rotation (upper right) or varus (lower left). A highly significant increase ($p < 0.001$) in valgus laxity (lower right) immediately after injury is still significantly increased at one and three weeks ($p < 0.02$ and $p < 0.01$ respectively), but has returned to normal by six weeks.

Fig. 3

Changes in the ultimate tensile strength of the MCL. For each time interval the mean percentage strength $\pm$ 2SE is shown in relation to normal values. The highly significant reduction ($p < 0.001$) observed immediately after injury has not changed by one week. At three weeks a significant increase ($p < 0.02$) has occurred, but still remains significantly reduced ($p < 0.01$) from normal. By six weeks strength has returned to normal levels.

post-injury and one week means ($p < 0.02$). After six weeks mean strength was 105.5%, no longer significantly different from controls.

Analysis of the elongation/load curve for the injured ligaments showed that compliance (Tan Q) was significantly less ($p < 0.02$) in the injured ligaments immediately after injury, and also at one and three weeks, but by six weeks there was no significant difference from controls ($p < 0.4$) (Fig. 4).

Macroscopic appearances. There are three components to the normal sheep MCL; the outer component extends from the femoral epicondyle to a diffuse insertion on the medial aspect of the tibia, well distal to the joint. Two short inner bands attach the meniscus to the femur proximally (meniscomfemoral ligament) and the tibia distally (meniscotibial ligament). This is very similar to the human MCL. On the outer surface of the ligament

Fig. 4

Changes in compliance of the MCL (tan Q). The mean value $\pm$ 2SE is given for each time interval. There is a significant decrease immediately after injury and at one and three weeks ($p < 0.02$). By six weeks compliance has returned to normal.

there is a very loose fibrous sheath containing some adipose tissue and blood vessels.

Immediately after injury mild haemorrhage was seen around the MCL, particularly towards the tibial insertion where there was some fraying and separation of its anterior border. The ligament was seen to have elongated and was slack when the medial joint surfaces were approximated. Rupture of the meniscomfemoral and meniscotibial parts of the ligament had occurred; the whole ligament could be separated from the medial meniscus. In two animals the joint capsule was torn anterior to the ligament. At one week the haemorrhage was resolving, but ruptured ligament fibres were still seen and, though capsular tears were not evident, the ligament was still free of the meniscus. By three weeks the outline of the ligament was obscured by thin, pearly-white scar tissue. The ruptured collagen fibres were no longer visible and in all specimens the ligament had become re-attached to the meniscus. At six weeks the tibial portion of the ligament was almost completely obscured by superficial scar tissue.

The cruciate ligaments were seen to be macroscopically intact in all specimens.

Histological appearances. *Normal ligament* (Fig. 5). This consists almost entirely of dense, closely packed, collagenous fibres orientated along the longitudinal axis of the ligament. Small spindle-shaped and darkly staining fibrocytes lie between the fibres. Blood vessels are rarely seen within the ligament.

Immediately after injury (Fig. 6). Sections through the tibial insertion site revealed rupture of superficial collagen fibres, particularly at the anterior aspect, with accompanying haemorrhage. All specimens showed rupture of the attachment of the ligament to the medial meniscus as evidenced by whorling and disorganisation of the fibres. Similar appearances were frequently observed in the superficial part of the tibial attachment. In all other areas the ligament appeared normal. In most specimens some extravasated blood was seen in the sheath.

One week after injury. In all ligaments there appeared to be a large increase in the cellularity along well-defined bands of the tissue. Cell counts within a fixed field confirmed this appearance. There was a very clear demarcation between these longitudinal bands of increased cellularity and the remainder of the ligament which appeared completely normal (Fig. 7). All of the "new" cells had the appearance of fibroblasts, being large, plump cells with large nuclei (Fig. 8). No inflammatory cells (neutrophils, macrophages or lymphocytes) were detected although some neutrophils were seen in the haemorrhagic areas of the sheath. The increased cell population was particularly evident superficially at the tibial insertion and in the areas adjacent to the meniscus which were seen to be damaged immediately after injury, but the precise sites of these fibroblastic bands in other parts of the ligament varied

between specimens. There were some short, discrete, isolated collagen bundles that were deeper staining and less cellular than the surrounding tissue; we interpreted these as being original but damaged collagen yet to be resorbed. Occasional thin walled blood vessels were seen within the damaged tissue.

Three weeks after injury (Fig. 9). Increased cellularity was still evident in the areas of repair. However there was a spectrum of cell morphology ranging from plump to elongated with deeper staining nuclei. New collagen fibres were not orientated in a uniform direction. Remnants of old collagen bundles were no longer seen and blood vessels were present in increased numbers.

Six weeks after injury (Fig. 10). Higher than normal cellularity was maintained, though the cells were now uniformly spindle-shaped and embedded in dense collagen orientated along the longitudinal axis of the MCL. There were still some isolated sites of active fibroblastic reaction and occasional blood vessels were still present.

DISCUSSION

In clinical terms, the lesion produced in this study was a Grade II ligamentous injury. That is, manipulation immediately after injury showed a marked increase in valgus laxity, but with a firm end-point.

Fig. 5

Fig. 6

Fig. 7

Figure 5 – Normal ligament. Darkly staining spindle-shaped fibrocytes are seen between closely packed, longitudinally aligned collagen fibres. The “feathering” and gaps are artefacts (H&E x 25). Figure 6 – Immediately after injury, showing rupture of the collagen fibres near the tibial insertion (H&E x 20). Figure 7 – At one week, showing the sharp demarcation between normal ligament on the left of picture and the hypercellular, damaged ligament to the right (H&E x 20).

Fig. 8

Fig. 9

Fig. 10

Figure 8 – At one week, a higher power view from the same region as Figure 7, shows the cells to be fibroblasts. There are no acute inflammatory cells (H&E x 40). Figure 9 – At three weeks, to show the spectrum of cell morphology. Adjacent to the normal ligament on the far left, mature spindle-shaped fibrocytes are seen amongst longitudinally aligned collagen fibres. Further to the right, the cells are more plump and the collagen is not uniformly aligned (H&E x 20). Figure 10 – At six weeks the newly synthesised collagen is well orientated in the longitudinal axis of the ligament. The fibrocytes are more spindle-shaped than at three weeks, but there is still an abnormally high cell population (H&E x 20).

This is the first reported animal model of a reproducible Grade II ligament injury. Miltner and his co-authors (1933, 1937) produced "mild" and "severe" sprains of the knee and ankle joints of rabbits by manual force, but, like Jack (1948, 1950), who used cats, they found that the severity of the lesions was very variable. This inability to produce a consistent lesion that resembles a clinical injury has hampered research on sprained, as opposed to ruptured ligaments (Clayton and Weir 1959; O'Donoghue et al. 1961).

In our method, the production of a characteristic sound serves as an end-point to define the limit of applied external rotation. This indicator was necessary because of the wide range of the ultimate tensile strengths of the individual ligaments. Thus, had we applied a uniform load to all ligaments then very inconsistent lesions would have resulted.

The relatively long-term increase in valgus laxity compared with the short duration of the lateral rotatory laxity and the lack of an increase in varus or medial rotation, indicate that the tissue injury was isolated to the MCL. The initial reduction of mechanical strength to 13.3% of that of the control indicates the severity of the lesion, but the fact that some strength remained confirms that the ligament was not completely ruptured. The consistent site of disruption of collagen fibres at the anterior aspect of the tibial insertion, and the rupture of the meniscomfemoral and meniscotibial components, both confirmed histologically, indicate that damage had spread throughout the whole ligament.

The residual strength must lie in the clearly defined tracts of the ligament that remain histologically undamaged. These tracts may be a reflection of the mechanical functioning of the ligament. Frisden et al. (1969) postulated that a ligament consists of individual elements equivalent to springs of different lengths which are gradually recruited to resist an increasing tensile load. This offers an explanation for the non-linear stress-strain relationship that is characteristic of ligaments.

Healing of soft-tissue injuries is usually mediated by an inflammatory response whereby the ingrowth of vascular channels bring both phagocytic cells to digest necrotic and damaged tissues and fibroblasts to lay down scar tissue. However, we found no migrating inflammatory cells within the ligament, an indication that this is not an inflammatory response. The repair and regeneration appeared to be mediated by fibrocytes.

From our study it is not possible to determine the actual origin of the observed fibrocytes. As no exogenous cells such as neutrophils or macrophages were seen within the damaged areas of the ligament, we believe that the fibrocytes originate from within the ligament by replication of the normally quiescent fibrocytes of the ligament.

Gamble, Edwards and Max (1984), immobilised rabbits' knees and noted the appearance of irregular and more widely-spaced ligament fascicles at four and eight

weeks, without any microscopic evidence of an inflammatory reaction. They believed that the changes were not due to the action of phagocytic cells originating outside the ligament, but stopped short of stating that the intrinsic fibrocytes were responsible. Such prolonged immobilisation has also been shown to reduce dramatically the ultimate tensile strength of ligaments (Noyes et al. 1974; Noyes 1977).

Frank, Amiel and Akeson (1983) discovered that after transection of the rabbit MCL there was a very sharp decline in collagen content in the first 20 days, followed by a slow increase. The content of immature collagen rose steeply up to 40 days after injury then declined equally steeply to 98 days. Thus, a high level of collagen degradation was occurring at the same time as an increased synthesis of new collagen. These workers found that the various biochemical parameters had still not returned to normal levels after 14 weeks. This correlates well with the histological and mechanical results of our study, with discrete bundles of deeply staining necrotic collagen in damaged areas at one week. At three weeks these were no longer seen, but fine, newly synthesised collagen bundles had appeared.

There have been many experimental studies in which ligaments were simply transected without any tensile damage elsewhere in the ligament (Horwitz 1939; Clayton and Weir 1959; O'Donoghue et al. 1961; Kappakas et al. 1978; Arnoczky et al. 1979; Cabaud et al. 1979; Fukubayashi et al. 1980; Frank et al. 1983; Korkala, Rusanen and Grönblad 1984). This injury is not a true replication of the condition seen in human patients in which the ligament is rarely cleanly severed when not loaded. For this reason the clinical relevance of such studies must be questioned, especially as our study has shown that extensive damage occurs along the whole length of the ligament before rupture.

In summary, our study has revealed that the strength of the MCL can be reduced to 13% of normal and yet still provide a firm end-point on physical examination. This should be taken into account when examining the acutely injured knee.

Furthermore, we have shown that there is a fundamentally different repair process for the Grade II ligament injury compared with that seen after the sharp transection of the Grade III lesion. We now believe that ligaments heal by an intrinsic fibroblastic mechanism.

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