Ligament grafts become more susceptible to creep within days after surgery

Evidence for early enzymatic degradation of a ligament graft in a rabbit model

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ABSTRACT – Clinical evidence suggests that some ligament grafts stretch after surgery. Our purpose in this study was to quantify early postoperative creep behavior of ligament autografts in an animal model, and to explore potential mechanisms of that behavior. 38 New Zealand white rabbits underwent a unilateral, fresh, anatomic medial collateral ligament (MCL) autograft procedure and were killed immediately (time-zero), at 2 days, 3 weeks, or 8 weeks after surgery (n = 7–11 in each group). We compared the creep behavior of the autografts to normal MCLs (n = 8). An additional 7 MCL specimens were incubated for 2 days in a low concentration collagenase solution and then similarly creep-tested. All grafts were slower to recover their original length after creep than either normal ligaments or time-zero controls. These grafts started to become more vulnerable to elongation in cyclic and static creep tests within 2 days of surgery, compared to time-zero controls. This vulnerability to creep increased over the next 3 weeks, and was maintained at 8 weeks of healing. 2-day collagenase-soaked MCL specimens had the same creep strains as the 2-day autografts. These results suggest that even fresh anatomic ligament autografts become vulnerable to creep within a few days after surgery by mechanisms that may involve degradative enzymes such as collagenase.

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Surgical reconstruction of ligament-injured joints using soft tissue grafts has become common (Noyes et al. 1983, Howe et al. 1991, Aglietti et al. 1992). Sometimes these procedures fail to provide lasting joint stability (Daniel and Reihl 1990, Aglietti et al. 1992, Lerat et al. 1997). Evidence suggesting that virtually all such grafts become less stiff and weaker than they were at the time of surgery has led to the assumption that it is only these two structural properties which define graft outcome (Holden et al. 1988, Kasperczyk et al. 1993, Ng et al. 1995).

Another possibility is that some grafts could fail by a mechanism of progressive elongation (called unrecovered creep) in response to repetitive tensile loads during joint function (Holden et al.1994). There is considerable clinical evidence in the literature concerning the anterior cruciate ligament (ACL) to support the concept that some grafts probably creep irreversibly. To prevent such potential graft "stretching out", one must examine its mechanisms.

In this study, we used the fresh anatomic medial collateral ligament (MCL) autograft model to define and quantify the susceptibility of ligamentous grafts to creep. We aimed to gain an understanding of how an early increase in creep becomes apparent. We have previously reported that these MCL autografts are more susceptible to creep 3 weeks after surgery (Boorman et al. 1998). The potential mechanisms responsible for this observation include scar infiltration into the graft, increased water content, and enzymatic degradation. In this in vitro study, graft creep was quantified immediately after surgery (time-zero)

and after healing for 2 days. We speculated that if grafts were found to be more vulnerable to creep within days after surgery, this would provide evidence that degradative mechanisms would be at least partially responsible. We also aimed to investigate the effects of short-term, low concentration collagenase exposure on the creep response of the MCL, to give us further insight into potential mechanisms for the biomechanical deterioration of the grafts.

Methods and material

Study design

22 skeletally mature (> 12 months), female New Zealand white rabbits (weighing 4.5–6 kg) underwent a standardized orthotopic medial collateral ligament (MCL) autograft procedure on the right hind-limb, using a previously described method (Sabiston et al. 1990). 28 of the 22 hind-limbs were used for mechanical creep testing (n = 7 at time-zero, and n = 11 after 2 days of healing). The remaining 4 grafted hind-limbs were used to measure graft water content after 2 days of healing, and the results were compared to normal MCL (n = 6). 5 more animals were used to assess other potential mechanisms for mechanical deterioration of graft tissue. 9 hind-limbs were used for these low concentration collagenase experiments (n = 7 for creep testing, n = 2 for water content).

We also compared the results of the current experiments to the creep results of: 1) the normal rabbit MCL, 2) 3-week MCL autografts, and 3) 8-week MCL autografts, previously reported (Boorman et al. 1998).

Autograft model

The MCL was harvested with femoral and tibial bone plugs, which had been predrilled and pretapped before removal of the graft. This was removed, washed in saline, and replaced immediately with screw fixation in an anatomical position (Sabiston et al. 1990). The animals were allowed normal activities in a 65 × 45 × 30 cm cage, and they received a standard diet and water ad libitum. They were handled according to established ethical guidelines approved by the local animal care committee. Some were killed with a phenobarbitol overdose at time-zero (n = 7) and others at 2 days (n = 11) after surgery. The hind-limbs were harvested, and frozen with the skin intact, in a sealed plastic bag at –70 °C.

Mechanistic factors

In addition to the time-zero and 2-day grafts used to assess creep potential, two other groups were included to study the potential mechanisms of graft creep vulnerability. Four 2-day grafts were examined for water content, without being subjected to creep testing. 9 MCLs were treated with collagenase for 2 days: 7 of these were creep-tested and the remaining 2 were exmained for water content without creep testing. After the dissection described previously, specimens were immersed in buffered collagenase at a concentration estimated to be physiologic for the rabbit MCL (Harper et al. 1988). 4 units/mL of bacterial collagenase was mixed in buffer containing 0.15 mM NaCl, 4mM $CaCl_2$, 50 mM Tris-HCl and 50 µg/mL gentamicin at pH 7.4. Specimens were incubated for 2 days at 37 °C. To assess tissue water content, the ligament or graft was excised and lyophilized until the dry weight stopped changing. Water content was the difference between the wet and dry weight divided by the wet weight expressed as a percentage.

Biomechanics

On the morning of testing, the samples were thawed at room temperature and all soft tissues were removed from the knee apart from the collateral ligaments, the cruciate ligaments, and the menisci. The bones were then cut with a saw about 5 cm from the joint line. These early grafts were handled in a fashion identical to those tested at 3 weeks (n = 10) and 8 weeks (n = 10), as reported previously (Boorman, et al. 1998). Likewise, normal MCL creep data collected in this previous study were used for comparison with the grafts (n = 8).

The dissected samples were potted in polymethylmethacrylate, and mounted on a specialized closed loop, servo-hydraulic material testing system (MTS Systems Corporation, Minneapolis, Minnesota) for creep testing. After mounting the tibia of the specimen in series with a load cell on the actuator cross-head and aligning the length of the graft with the load axis of the testing machine,

the femur (at a joint angle of 70° flexion) was lowered into a second pot and fixed similarly with cement. Both the femoral and tibial graft bone plugs were incorporated into the cement to avoid slipping at the fixation sites. The cement was still kept away from the ligamentous portion of the graft, thus avoiding thermal damage from exothermic curing. During mounting, the grafts were kept moist by frequent irrigation with 0.9% phosphate buffered saline.

The lateral collateral ligament, cruciate ligaments and the menisci were removed, leaving the femur-MCL-tibia complex. This complex then underwent 2 cycles between 2 N of tension and 5 N of compression to gain a measure of "ligament zero" which was determined to be the cross-head position at which the ligament began to take up a detectable load (0.1 N of tension) (Sabiston et al. 1990). The ligament length was measured at this position in a standardized way, using digital calipers. The medial femoral condyle was then carefully removed and special calipers used to measure the cross-sectional area of the grafts at the joint line (accurate to ± 5%) (Shrive et al. 1988). Cross-sectional area measurements taken at the joint line were used to calculate the force necessary to apply the same average stress of 4.1 MPa to each specimen. The 4.1 MPa stress level is about 5% of the failure stress of a normal rabbit MCL, and represents the estimated normal tensile loads borne by the rabbit knee ligaments in vivo (Sakane et al. 1997). The loads required were as follows: 2-day grafts = 21 (SD 5.2) N (n = 11), time-zero grafts = 15 (SD1.9) N (n = 7), and normal MCL controls = 16 (SD 1.8) N (n = 8). We have previously found no difference in the creep of normal rabbit MCLs between the toe region stress levels of 4 MPa and 7 MPa (Thornton et al. 2000), representing larger differences in load on the ligaments than in this experiment (4–5.4 MPa, based on a normal cross-sectional area). Thus, the various loads in these experiments would not be expected to cause differences in creep. Furthermore, the loads used in this experiment were thus standardized for differences in cross-sectional area. After measuring the cross-sectional area, each specimen was enclosed in a humidity chamber (relative humidity 99%) at 37 °C, to provide a constantly, moist environment during creep testing (Wilson et al. 1995).

The creep protocol entailed 30 cycles of cyclic loading at 1 Hz. to a constant stress level of 4.1 MPa, followed immediately by a 20-minute static creep, at the same stress level. The resulting elongation of each graft was measured by cross-head displacement and stored on a computer data file (Compaq 486). Tensile strain was defined as this measured graft deformation, divided by the ligament length measured at "ligament zero". Cyclic creep strain was the increase in strain from the peak of the first cycle to the peak of the thirtieth cycle. Static creep strain was defined as the increase in strain from the beginning of the application of stress to the end of the 20 minutes of constant stress (no recovery was allowed between the cyclic and static tests). Total creep strain (creep strain resulting from serial cyclic and static creep tests) was defined as the increase in strain from the peak of the first cycle of cyclic creep to the end of the static creep test. After the creep testing, the ligaments were allowed to recover at zero load for 20 minutes. The residual strain after this 20-minute recovery period was called unrecovered creep strain.

To check for potential creep occurring at the bone fixation sites of grafts before bony healing, magnified video images of creep tests were used to confirm no movement of bone grafts. To minimize further potential bony contribution to the creep strains measured, we also incorporated the bone grafts in the cement during potting, and thus reinforced their fixation. Finally, time-zero ligament grafts were creep tested, and found to be about the same as normal ligament controls. These results showed that no creep was occurring at the bone graft fixation sites during testing.

Statistics

We included our previous data (Boorman et al. 1998) in the statistical analysis, so that we could take advantage of the various healing times (time-zero, 2 days, 3 weeks, and 8 weeks); thus, outcomes were analyzed statistically using a one-way ANOVA, with time as the variable (SAS software version 6.12). The time-zero grafts, 2-day grafts, the collagenase-treated ligaments, and normal MCLs were also analyzed with a one-way ANOVA with treatment as the single variable. Groups were then compared using linear contrasts to obtain an

Summary of creep strain data, average strain. Values are percent (SD)

	XSA (mm^2)	Cyclic creep strain	Static creep strain	Total creep strain	Unrecovered creep
Normal MCL	3.94 (0.44)	0.17 (0.17)	0.71 (0.13)	0.99 (0.20)	0.70 (0.23)
Time-0 graft	3.64 (0.47)	0.20 (0.03)	0.69 (0.10)	1.05 (0.10)	0.44 (0.38)
2-day graft	5.19 (1.31)	0.42 (0.15)	0.86 (0.19)	1.29 (0.32)	1.12 (0.29)
2-day collagenase	5.15 (1.12)	0.38 (0.08)	0.76 (0.18)	1.27 (0.22)	1.02 (0.22)
3-week graft	5.87 (0.74)	0.51 (0.12)	0.98 (0.19)	1.63 (0.32)	1.19 (0.01)
8-week graft	7.07 (0.56)	0.59 (0.11)	1.02 (0.16)	1.74 (0.25)	1.22 (0.23)

exact p-value for each comparison. A significance level of 0.05 was used in all tests.

Results

Gross morphology and cross-sectional area

Even 2 days after transplantation, the MCL autografts were visibly different from the normal MCLs. They had a fibrovascular scar material adhering to their surface. The graft fixation sites remained firmly attached by the screw. After healing for 3 weeks we found abundant new material encasing the MCL autograft and knee joint. This scar was fibrous. Similar scarring was seen at 8 weeks. At both 3 and 8 weeks, the graft fixation sites showed substantial bony healing.

The grafts increased in cross-sectional area with time (ANOVA; $p = 0.0002$) (Table). The 2-day grafts were larger than the time-zero ones and normal MCL controls ($p = 0.001$ and $p = 0.0003$, respectively).

Biomechanics

After 2 days of healing the MCL autografts crept during cyclic creep testing more than the normal MCLs and the time-zero control grafts ($p = 0.0003$ and $p = 0.001$, respectively) (Figure 1). We also found an increase in mean graft cyclic creep strain during healing (ANOVA; $p < 0.0001$) (Figure 2). No difference was noted between the time-zero control grafts and the normal MCLs ($p = 0.7$) (Figure 2).

In the static creep strain tests, the 2-day grafts crept more than the time-zero ones ($p = 0.03$) and the normal MCL controls ($p = 0.04$). The vulner-

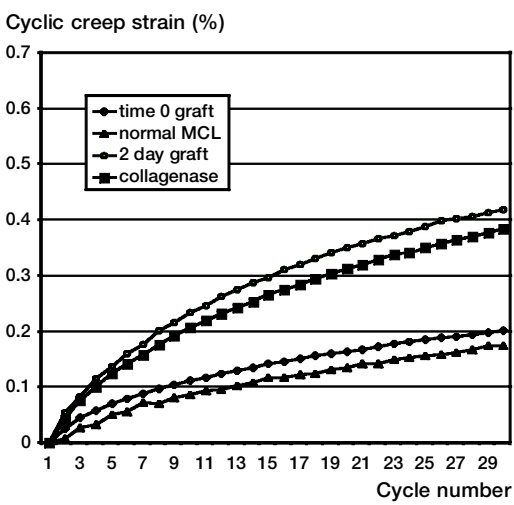

Figure 1. Cyclic creep strain %. Group means for each cycle. Specimens were repeatedly stressed to 4.1 MPa at a frequency of 1 Hz.

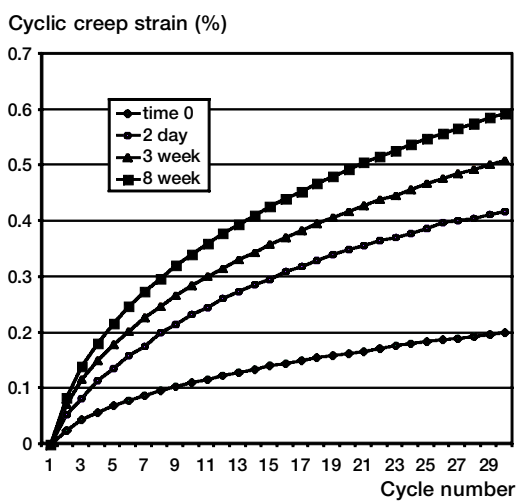

Figure 2. Cyclic creep strain % of MCL grafts over 8-week healing interval. The increase in cyclic creep strain with time was statistically significant ($p < 0.0001$).

ability of the grafts to static creep increased further at the same stress with time ($p = 0.002$) (Table).

The total creep of the grafts represents the cumulative increase in strain resulting from serial cyclic and static creep tests. The 2-day grafts crept more than the time-zero controls ($p = 0.04$) and the normal MCLs ($p = 0.01$) (Table). We also saw an increase in graft creep over the 8-week healing interval ($p < 0.0001$). No difference was found in the total creep strain between normal MCLs and time-zero controls ($p = 0.7$).

The 2-day grafts were less able to recover their original length than the time-zero grafts ($p < 0.0001$), and the normal MCL controls ($p = 0.004$). No differences were detected in the unrecovered creep strain with time. In the test conditions used, the normal MCLs and the time-zero control grafts did not return to zero strain (Table).

Mechanistic factors

Although differences were found between the creep parameters of 2-day grafts and normal MCLs, the water content of the 2-day grafts (68 (SD 0.3)%, $n = 4$) was about the same as the normal water content of the MCL (66 (SD 3.2)%, $n = 6$). Likewise, no statistically significant difference was found between the normal MCLs and those soaked in collagenase for 2 days (70 (SD 0.7%), $n = 2$).

The cyclic creep strain (Figure 1) and the total creep strain (Table) were greater in the collagenase-treated specimens than in the normal ligament controls ($p = 0.003$ and $p = 0.03$, respectively). On the other hand, we found no difference between the 2-day autografts and the collagenase-treated specimens as regards the creep parameters measured.

Discussion

We have previously reported that 3-week MCL autografts were more susceptible to creep than normal MCL controls (Boorman et al. 1998). The current study shows that the postoperative vulnerability of these fresh, anatomically placed, extraarticular rabbit MCL autografts to increased creep can be detected even 2 days after surgery. An increase was found in the cyclic, static, and total creep strain of the 2-day autografts, as compared to time-zero grafts and normal MCL controls. These results were seen even in this test of short duration (less than 30 minutes of total loading time), and under what can be estimated as low physiological loads (4 MPa). We found that in this model, the first days and weeks are most critical to the graft mechanical deterioration observed, since we noted no subsequent increase in the total creep strain of the 8-week grafts, as compared to the 3-week grafts (Boorman et al. 1998). Furthermore, all grafts were less able to recover their original length than normal MCLs during these test conditions. Because of this early increase in susceptibility to creep, and a relative inability of grafts to recover at the same rate as normal ligaments, we speculate that permanent stretch of the grafts could occur in vivo, particularly if the grafts were exposed to higher loads over longer periods of time.

These three mechanisms might explain the increase in graft creep: an increase in the water content of the graft, degradation of the graft by inflammatory enzymes, or scar tissue infiltration into the grafts. We evaluated the possible contribution of these factors.

Water content has been shown to be important in soft tissue mechanics, particularly as it relates to viscoelastic properties (Haut and Powlison 1990, Woo et al. 1993). Woo et al. (1993) speculated that interstitial water reduced friction between collagen fibers and favors interfibrillar sliding with tissue elongation. It makes the tissue less stiff and more viscoelastic (Woo et al. 1993, Hannafin and Arnoczky 1994). However, the water content of the 2-day grafts (68%) was about normal (66%). It therefore seems unlikely that this early creep susceptibility is due to an increase in water content alone.

Graft degradation therefore can be a second, and more plausible explanation of our results. A very rapid degradation of the collagenous scaffold may have been largely responsible for reducing the grafts' ability to resist creep loading at the 2-day healing interval. Rat Achilles tendon grafts have a rapid turnover of collagen (degradation and synthesis), with about 50% of the collagen being degraded in the first month (Klein and Lewis 1972). In our study, incubating the normal MCL for 2 days in a low concentration of collagenase caused an increase in creep of about the same extent as that of the MCL autograft that was allowed to heal for 2 days. This supports the view

that degradative processes may contribute to the early vulnerability of grafts to creep.

A third possible explanation of increased creep is that grafts are known to be infiltrated and partially replaced by what appears to be scar tissue (Amiel 1990). Biopsies of human soft tissue grafts (Oakes 1993) and animal ligament reconstructions (Ballock et al. 1989) have shown an almost complete cellular necrosis of the graft fibroblasts, followed by new blood vessel and scar tissue infiltration into the graft matrix. This would become a more important factor at the 3-week and 8-week healing intervals, but it is unlikely that substantial scar infiltration had occurred at 2 days after surgery. Recent studies have shown that scar tissue creeps more than normal ligament tissue for various reasons (Thornton et al. 2000). Therefore, the replacement of the degraded graft collagen scaffold by scar tissue would theoretically make the structure more susceptible to creep, even if the overall collagen balance is not negative, as has been previously shown (Klein and Lewis 1972).

The cross-sectional areas of the grafts had increased at only 2 days after surgery, and they continued to increase during 8 weeks. This finding may seem difficult to explain at 2 days, especially since the grafts had about the same water content. The 2-day grafts had granulation tissue on the surface that was carefully removed before testing. However, to avoid damaging the ligament, small amounts of this tissue may have been left in place, contributing to the slightly increased cross-sectional area. We speculate that the other possible mechanism for the increase in cross-sectional area at 2 days is related to enzymatic degradation of interfibrillar connecting collagen, allowing for a less tightly packed structure and a corresponding increase in cross-sectional area.

A main question, of course, concerns the relevance of these results to other grafts, including the ACL. As noted above, based on the early postoperative return of joint laxity in many ACL reconstruction models (usually within weeks) (Jackson et al. 1987, Lerat et al. 1997), some rapid mechanism of graft loosening must be present. These include slipping of the graft fixation, partial graft rupture, fatigue failure, fretting failure where the graft rounds a corner, secondary whole joint changes, or "elongation" of the entire graft. Ng et al. (1995) reported that the large increase in joint laxity observed in a goat anterior cruciate ligament reconstruction model only 3 weeks after surgery may be due to graft elongation secondary to an increase in the load relaxation behavior of the graft. Although load relaxation is a measure of viscoelastic behavior of soft tissues, it cannot be used to explain directly in vivo graft elongation (Holden et al. 1994). Our results support the concept that such ACL grafts may elongate in the early postoperative period, but via a mechanism of creep.

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