Electron microscopic evaluation of the effects of stress-shielding on maturation of the mid-substance and ligament-bone junction of the reconstructed anterior cruciate ligament in rabbits

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To analyze the effects of stress-shielding on graft maturation after the anterior cruciate ligament (ACL) reconstruction, autogenous ACL reconstruction using Achilles tendon was performed in rabbits. Two-end fixation with a ligament augmentation device (LAD), as a stress-shielding model (SS group), and pull-out fixation with Leeds-Keio artificial ligaments (L-K ligament), as a non-stress-shielding model (non-SS group), were investigated. Intact ACL was used as the control. Specimens were harvested 6 mon postoperatively, and the analysis was focused on collagen fibril maturation in the mid-substance and bone-anchoring pattern in the bone tunnel, under light and transmission electron microscopy. The density of collagen fibrils in the control was lower than that in both experimental groups ($p < 0.01$). The per cent collagen area in the control was higher than that in the SS ($p < 0.01$) and the non-SS group ($p < 0.05$). The per cent collagen area in the non-SS group was higher than that in the SS group ($p < 0.05$). At the ligament-bone interface, intracellular calcification of the degenerated chondrocytes occurred in the non-SS group, while in the SS group hydroxyapatite deposits were observed only in the extracellular matrix. These results show unfavorable influence of stress-shielding on graft maturation not only in the mid-substance but also at the ligament-bone junction. © *1999 Kluwer Academic Publishers*

1. Introduction

Among the knee-joint ligaments, the anterior cruciate ligament (ACL) plays an important role in both supporting and controlling the knee-joint movements, especially in twisting and pivoting. When it is injured, it results in serious functional impairment affecting particularly the practice of sports. Therefore, various reconstruction procedures [1–5] have been attempted to repair the injured ACL. Currently, reconstruction with bone- patellar tendon- bone (BTB) is considered as the gold standard. However, some unfavorable influences on the extensor mechanism of the knee have been reported when using this method [6–9]. So, grafting using autogenous semitendinosus and gracilis tendon as an alternative substance has been attempted for ACL reconstruction. However, in the case of tendon grafting, the fixation method is complicated because there are few appropriate procedures to fix the tendons rigidly [10]. To overcome this problem and make rehabilitation safer and faster, we developed and investigated an augmentation method using a ligament augmentation device (LAD, 3M Company, St Paul, MN) [11]. The effects of stress-shielding with such an artificial material have been widely discussed, however, they still remain to be well analyzed. It is well known that although two-end fixation provides a more rigid initial structure, it will result in more stress-shielding of the graft than a one-end fixation method. However, there are few statistical analyses of the maturation of collagen fibrils in the grafted tendon that evaluate the influence of stress-shielding on the reconstructed ACL. On the other hand, it has been reported that the reconstructed ligament heals both within the knee joint and the bone tunnel, following a common biological process [12–19]. However, the anchoring process at the ligament-bone interface under the influence of stress-shielding remains to be investigated. We designed a stress-shielding model using the LAD with a two-end fixation method in rabbits to answer such questions. We focused on not only collagen fibrils maturation in the mid-substance but also on the bone-anchoring pattern at the bone-ligament interface using transmission electron microscopy.

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2. Methods

Fifteen Japanese White adult male rabbits (weight 3.0 3.5 kg) were used. The operations were all performed under intravenous pentobarbital anesthesia. In five rabbits, the 3 mm wide LAD was wrapped around with the autogenous Achilles tendon taken from the right limb and sutured together with 3–0 Surgilon (Davis Geck Co., Wayne, NJ) at 5 mm interval, both ends of which were passed through holes 2.7 mm diameter made in the tibial and femoral bones after the right ACL was totally resected, and fixed at the original attachment sites. The Achilles tendon was fixed with multifilament nylon sutures using 3–0 Surgilon and LAD with specially made metal plates on both ends (Fig. 1). This was the SS group as the stress-shielding model. In the other five rabbits, the looped Leeds Keio (L-K) ligament [20, 21] was sutured to both ends of a double-folded autogenous Achilles tendon taken from the right limb with 3–0 Surgilon. Each end of the L-K ligament was passed through the bone tunnel made as in the SS group, and fixed with a screw in accordance with the pull-out method at the original attachment sites (Fig. 1). This was the non-SS group as the non-stress-shielding model for usual ACL reconstruction. No operative procedures were performed on the knees of the remaining five rabbits as a normal control. Animals in all groups were kept in the cage without immobilization throughout the experimental period.

Six months after surgery, the rabbits were sacrificed with a fatal dose of pentobarbital. The reconstructed ACL was removed including the bone-ligament junction kept intact. Specimens were trimmed into small pieces, fixed in 2.5% glutaraldehyde, rinsed in 0.1 M cacodylate buffered solution, post-fixed in 2.0% tetroxide osmium in the same buffered solution, dehydrated through a graded ethanol series, cleared in propylene oxide, and embedded in Epon 812. Thin sections were prepared and stained with toluidine blue for observation under a light microscope, and ultrathin sections were double-stained in uranyl acetate and lead citrate for examination with a transmission electron microscopy (Hitachi H-600, Tokyo, Japan).

In addition, each ultrathin section taken from the mid-substance of the reconstructed ACL at the central part was raised on copper 150 mesh grids, and electron micrographs of three randomly selected windows were taken at an original magnification of $\times 150\,000$ to measure the density (the number of collagen fibrils per square micrometre), the per cent collagen area (percent area occupied by total collagen fibrils relative to the cross-sectional area), and the diameter of collagen fibrils. Analysis was performed on a Macintosh model computer using the public domain NIH Image program (written by Wayne Rasband at the US National Institutes of Health and available from the Internet by anonymous ftp from Zippy.nimh.nih.Gov. or on floppy disk from NTIS, 5285 Port Royal Rd., Spring-field, VA22161, part number PB93-504868), and the differences were tested for statistical significance by Mann-Whitney test. The level of significance was $p < 0.05$.

3. Results

3.1. Measurement of collagen fibrils in the graft

3.1.1. Distribution of the collagen fibril diameter (Fig. 2)

In the control group, small- (<60 nm), middle- (60–100 nm), and large-diameter (>100 nm) collagen fibrils were seen, and two patterns of collagen fibril distribution were found. One was that of a group of fibrils in which the peak diameter was between 20 and 80 nm, and took a gently-sloping distribution pattern (ACL1–3). The other was a group in which large-diameter collagen fibrils accounted for more than 35.8%, and took a dispersed distribution pattern (ACL4–5). On the other hand, in the SS- and non-SS groups almost all collagen fibrils were small, with a peak diameter between 20 and 60 nm. Small-diameter collagen fibrils accounted for 86.4%–

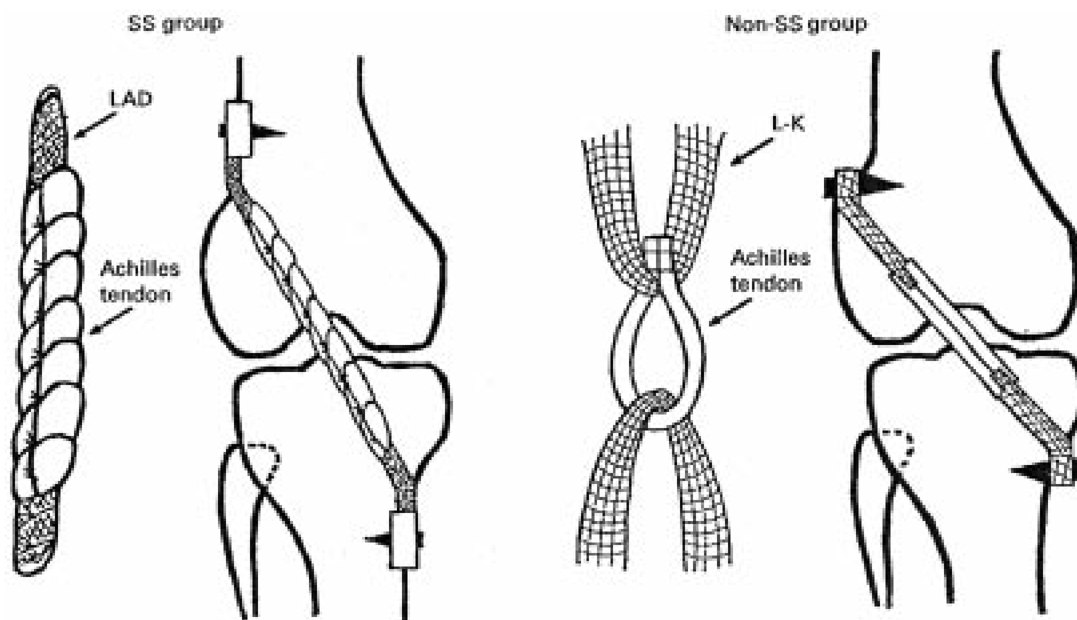


Figure 1 Operative procedure.

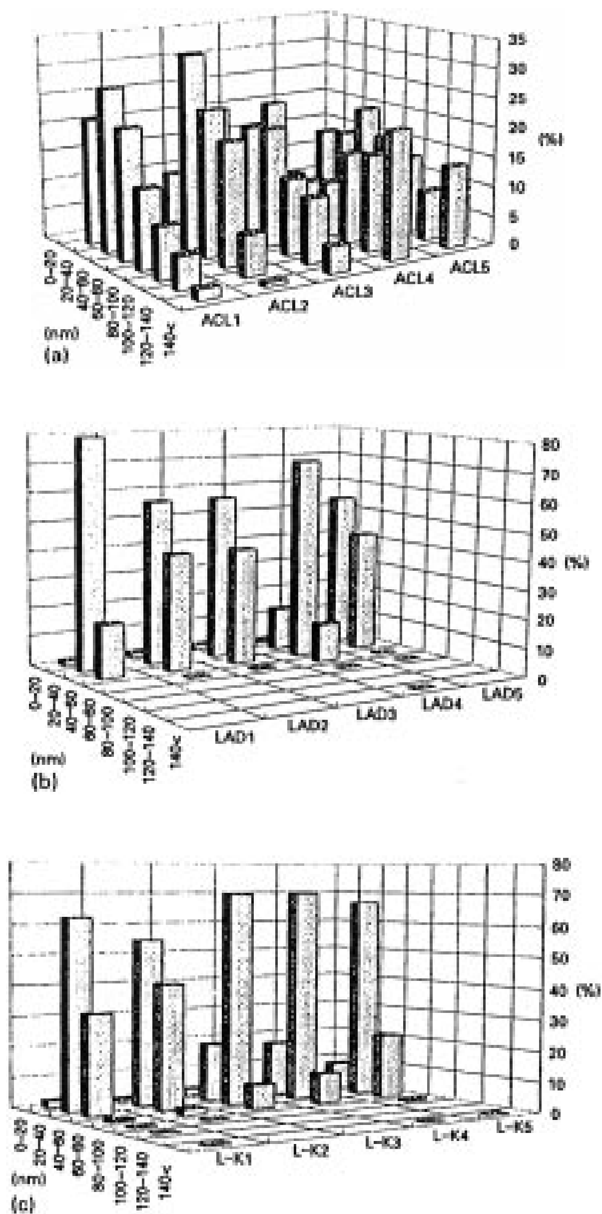


Figure 2 Distribution of the diameter of collagen fibrils of the graft. (a) Control group, ACL 1–5; (b) SS group, LAD 1–5; (c) non-SS group, L-K 1–5.

100% in the SS group, and 77.3%–98.0% in the non-SS group. The number of middle-diameter collagen fibrils in the non-SS group tended to be larger than that in the SS group, while large-diameter collagen fibrils accounted for 0%–0.2% in the SS group, and 0.3%–0.5% in the non-SS group. Very large-diameter (>140 nm) collagen fibrils were seen in one animal of the SS group (accounting for 0.2%) and three of the non-SS group (all accounting for 0.3%).

3.1.2. Collagen fibril analysis (Fig. 3)

The mean density of the collagen fibrils was $264.6 \pm 58.4 (\mu m^{-2})$ in the SS group and $225.1 \pm 55.5 (\mu m^{-2})$ in the non-SS group, and the density in both groups was significantly higher than the $88.3 \pm 26.4 (\mu m^{-2})$ observed in the control group ($p < 0.01$). The density in the SS group tended to be higher than that in the non-SS group, but there was no significant difference between them. The per cent

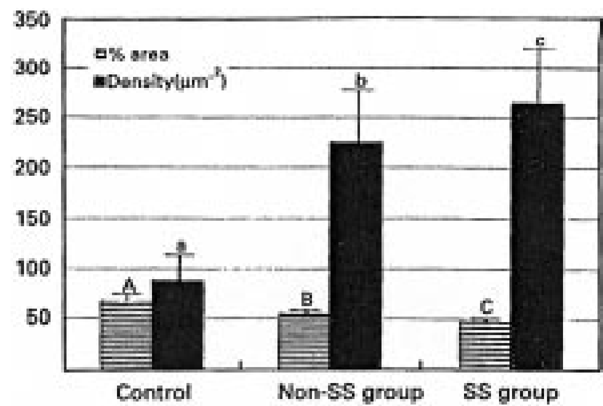


Figure 3 Results of the measurement of collagen fibrils of the graft. Per cent collagen area (per cent area occupied by total collagen fibrils relative to the cross-sectional area): $B < A (p < 0.05), C < A (p < 0.01), C < B (p < 0.05)$. Density (the number of collagen fibrils per square micrometre): $a < b (p < 0.01), a < c (p < 0.01)$.

collagen area in the control group was $66.5 \pm 7.6\%$; that is, significantly higher than the $47.4 \pm 3.5\%$ observed in the SS group ($p < 0.01$) and the $54.7 \pm 3.8\%$ in the non-SS group ($p < 0.05$). The per cent collagen area in the non-SS group was significantly higher than that in the SS group ($p < 0.05$).

3.1.2.1. Histology of the ligament-bone interface region.

(a) The SS group
(i) Light microscopic observation
Collagen fibers of the reconstructed ACL were arrayed in crimpy patterns at the entrance of the bone tunnel, but at the tendon-bone junction in the tunnel they became disarrayed, and the number of fibroblastic cells with an oval nuclei increased; incipient columniation of the fibrocartilage cells was formed. But there was no clear border (blue line) between the mineralized and non-mineralized fibrocartilage. In the center of the bone tunnel, only fibroblastic cells and collagen fibers were seen surrounding the fibers of LAD (Fig. 4).
(ii) Electron microscopic observation
In the hypertrophic layers of the chondrocyte column at the tendon-bone junction in the bone tunnel, chondrocytes contained many swollen mitochondria, secretory granules, and rough endoplasmic reticulum (RER) the cavities of which were irregularly expanded (Fig. 5a). All these findings indicate the characteristics of the activated

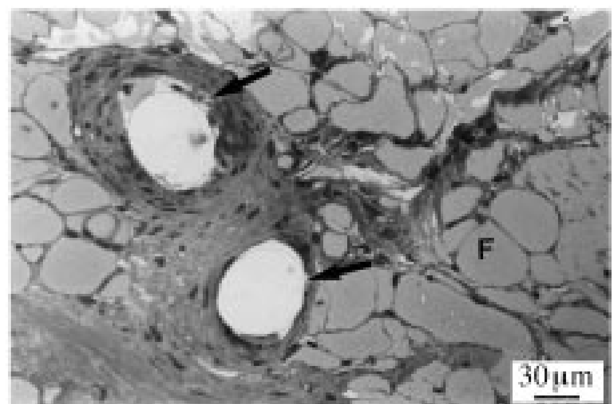


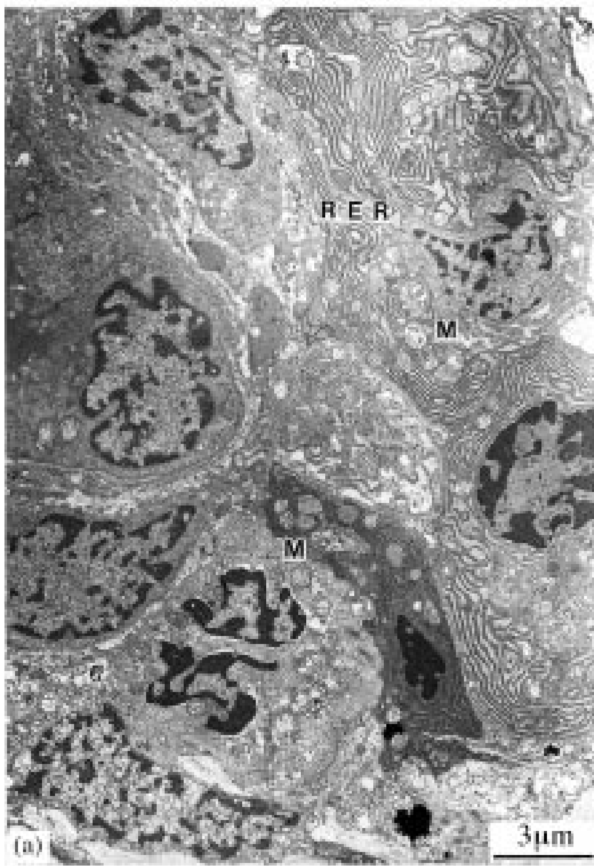
Figure 4 Light microscopic observation of the SS group. In the center of the bone tunnel, only fibroblastic cells and collagen fibers are seen surrounding the fibers of LAD (arrow). F, fat. Toluidine blue.

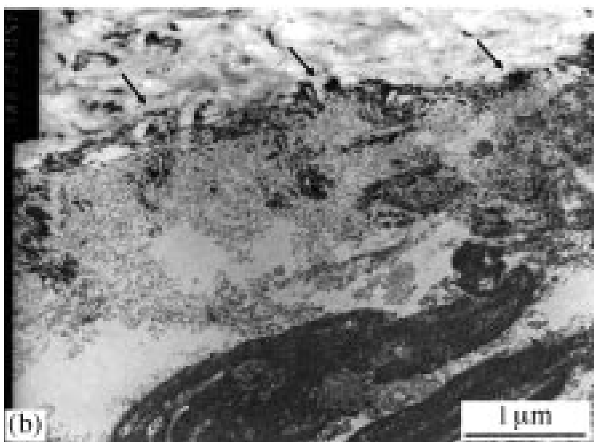
chondrocytes producing the extracellular matrix. The high-magnification view of the extracellular matrix in the layers of calcification revealed the presence of numerous needle-shaped crystals among the collagen fibrils (Fig. 5b). The results of electron diffraction analysis demonstrated these crystals were of hydroxyapatite (Fig. 5c). On the other hand, a large quantity of very thin collagen fibrils existed around the fibers of LAD through the bone tunnel. No chondrocytes were detected around the fiber of LAD.

(b) The non-SS group

(i) Light microscopic observation

In the knee joint, thick winding collagen fibers of the grafted tendon ran in parallel and fibroblastic cells with





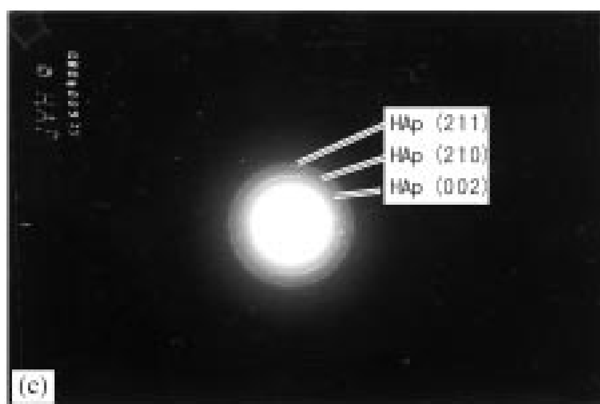
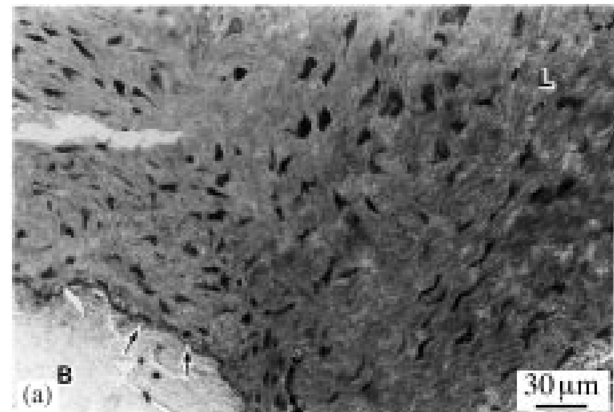


Figure 5 Electron microscopic observation of the SS group. (a) In the layers of hypertrophic cells, chondrocytes contain many swollen mitochondria (M) and rough endoplasmic reticulum (RER), the cavities of which are irregularly expanded. (b) In the layers of calcification, numerous needle-shaped crystals (arrow) are scattered among the collagen fibrils. (c) The result of electron diffraction analysis demonstrates these crystals are hydroxyapatite.



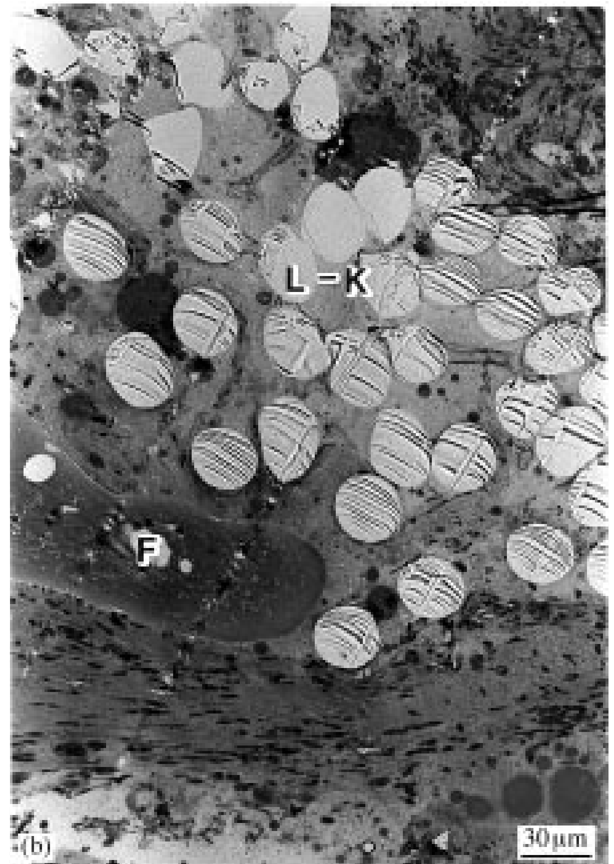


Figure 6 Light microscopic observation of the non-SS group. (a) At the grafted tendon (L) bone (b) junction in the bone tunnel, the number of cells having stellate nuclei becomes dominant and incipient columniation of the fibrocartilage cells (arrow) is formed. But there is no clear border (blue line) between the mineralized and non-mineralized fibrocartilage. (b) At the L-K ligament fixation sites in the bone tunnel, collagen fibers surround fibers of the L-K ligament (L-K), and chondrocytes are not observed. F, fat. Toluidine blue.

fine spindle-shaped nuclei appeared occasionally among them. At the tendon-bone junction in the bone tunnel, the number of cells having stellate nuclei became dominant, but columniation of the fibrocartilage cells was not well-developed, and the blue line separating unmineralized and mineralized fibrocartilage was not observed (Fig. 6a). At the L-K ligament fixation sites in the bone tunnel, collagen fibers surrounded fibers of the L-K ligament, and obvious chondrocytes were not observed (Fig. 6b).

(ii) Electron microscopic observation

At the tendon-bone junction in the hole, chondrocyte column formation was in its early stages, that is, fibrocartilage cells were aligned from a few layers of chondroblasts to matured chondrocytes (Fig. 7a). In the calcified layers, cells with obscure intercellular borders contained round, swollen RER, many secretory granules and crystal precipitations in the cytoplasm (Fig. 7b). These crystal precipitations corresponded to hydroxyapatite as demonstrated by electron diffraction analysis (Fig. 7c), suggesting calcification in the cytoplasm of the degenerated chondrocytes. On the other hand, at the L-K ligament-bone junction, small- and large-diameter collagen fibrils were observed around the L-K ligament fibers. Neither chondrocytes nor hydroxyapatite deposits were observed around the fibers of the L-K ligament. The density of the collagen fibrils around the L-K ligament fiber was thicker than that around the LAD fiber.

4. Discussion

We used a rabbit model to analyze the effects of stress-shielding, because in this model it was possible to analyze the whole grafted tendon, including the ligament- bone junction, while in a biospy specimen only the superficial layer from one site of the grafts can be evaluated. The analysis of the collagen fibrils showed that in the SS group the per cent collagen area was statistically lower and the density of collagen fibrils tended to be higher than in the non-SS group. The distribution pattern of collagen fibrils of different diameters in the non-SS group tended to shift towards the middle-diameter fibril compared to that in the SS group. These findings from the analysis on the whole grafts may indicate that stress on a graft tissue enhances the thickening of the collagen diameter, that is, makes the reconstructed ligament biomechanically stronger. Furthermore, at the grafted tendon-bone junction in the bone tunnel, calcification was noted in the cytoplasm of degenerated chondrocytes in the non-SS group, indicating chondrocytes maturation (last stage) with a normal four-layer structure [13]. In the SS group, however, insufficient maturation of the tendon bone interface up to intracellular calcification of the degenerated chondrocytes was confirmed, and numerous hydroxyapatite crystals were observed scattered among the extracellular matrix in the calcified layers. These findings in the SS group can be correlated with immature calcification at the tendon-bone interface in the bone tunnel, which suggests unfavorable influence of stress-shielding caused by fixation of the LAD on both ends. Furthermore, it is suggested that in the early stages of the grafted tendon-bone anchoring, hydroxyapatite deposits in the matrix vesicles among the

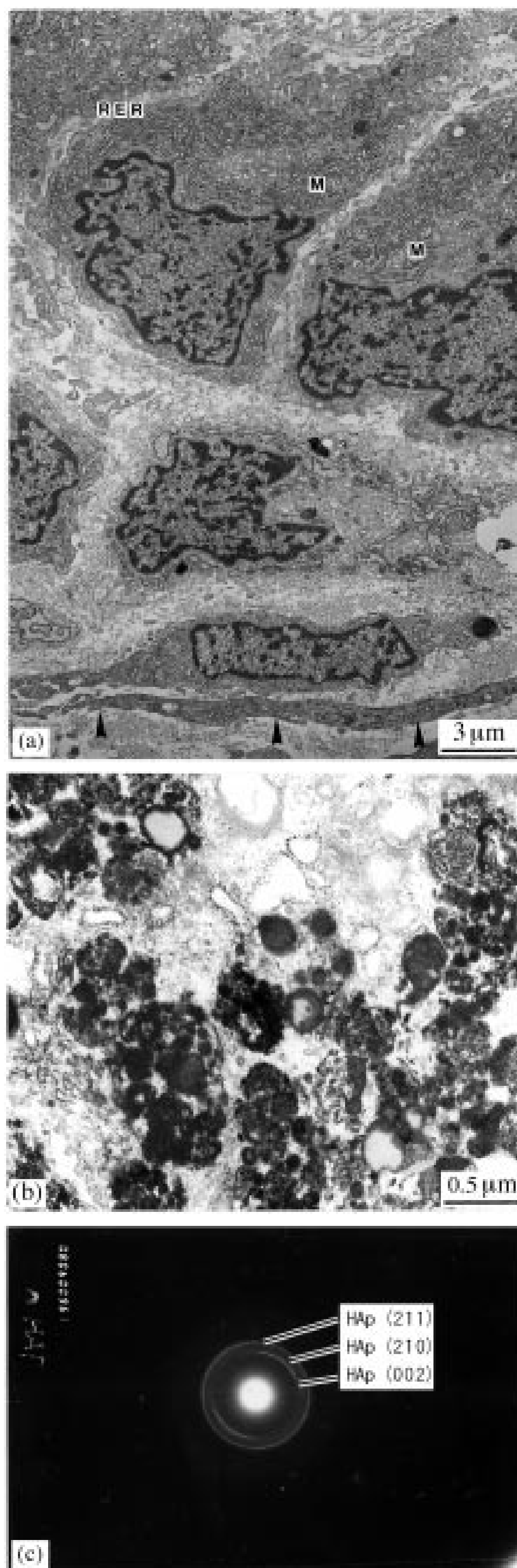


Figure 7 Electron microscopy of the non-SS group. (a) At the ligament- bone junction in the hole, chondrocyte column formation is in its early stages, that is, fibrocartilage cells are aligned from a few layers of chondroblasts (arrow-head) to matured chondrocytes. M. mitochondria. (b) Many crystal precipitations in the cytoplasm in the calcified layers. (c) These crystal precipitations correspond to hydroxyapatite, as demonstrated by electron diffraction analysis.

collagen fibrils only, but as the maturation of the tendon-bone anchoring progresses, hydroxyapatite deposits in the matrix vesicles formed among the remnants of cytoplasmic organelles of the degenerated or dead chondrocytes.

In both experimental groups the density of collagen fibrils was higher and the per cent collagen area was lower than that in the control group. In the control group collagen fibrils distributed continuously from small- to large-diameter fibrils, and large-diameter fibrils over 100 nm accounted for 15.7%–53.0%. However, large-diameter fibrils accounted for 0%–0.2% in the SS group and 0.3%–0.5% in the non-SS group, and in both experimental groups there was a gap between middle- and very large-diameter fibrils, which suggested that these very large-diameter collagen fibrils were degenerated remnants of the donor tendon. These findings indicated that the majority of collagen fibrils of the grafted autogenous tendon had been replaced by small-diameter fibrils, but a few fibrils remaining were still observed even at 6 mon after the operation, either with or without stress-shielding.

Oakes *et al.* [22] reported a quantitative collagen fibril analysis of the adult goat patellar tendon ACL autografts. They showed by electron microscopy that the large-diameter fibrils progressively disappeared, and that the small-diameter fibrils increased with time until 52 wk after grafting. In another study of ACL reconstruction using goat patellar tendon performed by Jackson *et al.* [23], the 24 wk autograft group demonstrated a large increase in the number of small-diameter fibrils which accounted for 84% of the fibril population. In spite of the difference in the observation period or activities after operation, the number of large-diameter collagen fibrils of more than 100 nm was very small at the final observation time in both studies. This fact may suggest limitation of the maturation of the reconstructed ACL.

Further investigations will be needed to find out whether the reconstructed ACL can ever regain its normal ''bimodal'' pattern of collagen fibrils when some favorable stress is applied on the graft, as well as to find out how long it would take for the reconstructed ACL to regain the number of larger fibrils, as seen in the normal ACL.

5. Conclusions

Reconstructed ACL without stress-shielding consisted of larger collagen fibrils than those with stress-shielding in the mid-substance, although both groups showed a unimodal pattern without large-diameter fibrils. The reconstructed ACL with stress-shielding showed limited maturation of the four-layer structure of the tendon-bone junction with a small area of extracellular calcification, while the reconstructed ACL without stress-shielding demonstrated mineralized fibrochondrocytes with massive hydroxyapatite in the cytoplasm of degenerative cells.

References

1. W. C. CAMPBELL, *Am. J. Surg.* **43** (1939) 473.
2. E. ERIKSSON, *Orthop. Clin. North. Am.* **7** (1976) 167.
3. K. JONES, *J. Bone Joint Surg.* **64-A** (1963) 925.
4. D. MACINTOSH, *ibid.* **56-B** (1974) 591.
5. L. E. PAULOS, D. L. BUTLER, F. R. NOYES and E. S. GROOD, *Clin. Orthop.* **172** (1983) 78.
6. R. A. HARTER, *Am. J. Sports Med.* **16** (1988) 434.
7. T. D. ROSENBERG, J. L. FRANKLIN, G. N. BALDWIN and K. A. NELSON, *ibid.* **20** (1992) 525.
8. K. SHINO, S. NAKAGAWA, M. INOUE, S. HORIBA and M. YONEDA, *ibid.* **21** (1993) 206.
9. K. YASUDA, *ibid.* **20** (1992) 471.
10. D. M. DANIEL, in ''Orthopaedic knowledge update 3'' edited by W. B. Greene, (AAOS, Rosemont, 1990) p. 557.
11. J. C. KENNEDY, *Clin. Orthop* **172** (1983) 125.
12. W. G. CLANCY, M. S. NARECHANIA, T. D. ROSEN-BERG, J. G. GMEINER and T. A. LANGE, *J. Bone Joint Surg.* **63-A** (1981) 1270.
13. R. R. COOPER and S. MISOL, *ibid.* **52-A** (1970) 1.
14. A. D. FORWORD and R. J. CORWAN, *ibid.* **45-A** (1963) 807.
15. M. HAUSMAN, S. BAIN and C. RUBIN, *Trans. Orthop. Res. Soc.* **14** (1989) 277.
16. G. A. KERNWEIN, *Surg. Gynec. Obstet.* **75** (1942) 794.
17. P. K. NIKOLAOU, A. V. SEABER, R. R. GLISSON, B. M. RIBBECK and F. H. BASSETT, *Am. J. Sports Med.* **14** (1986) 348.
18. S. A. RODEO, S. P. AMOCZKY, P. A. TORZILLI, C. HIDAKA and R. F. WARREN, *J. Bone Joint Surg.* **75-A** (1993) 1795.
19. T. B. WHISTON and R. WALMSLEY, *ibid.* **42-B** (1960), 377.
20. K. FUJIKAWA, in ''Prosthetic ligament reconstruction of the knee'' edited by M. J. Friedman and R. D. Ferkel (Saunders Philadelphia, PA, 1988) p. 132.
21. K. FUJIKAWA, F. ISEKI and B. B. SEEDHOM, *J. Bone Joint Surg.* **71-B** (1989) 566.
22. B. W. OAKES, M. KNIGHT, I. D. MCLEAN and O. DEACON, in ''Proceedings of AOSSM'', San Diego (1992) p. 45.
23. D. W. JACKSON, E. S. GROOD, J. GOLDSTEIN, M. A. ROSEN, P. R. KURZWEIL, J. F. CUMMINGS and T. M. SIMON, *Am. J. Sports Med.* **21** (1993) 176.

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