Bone-tendon-bone (B-T-B) type grafts were prepared for ligament reconstruction by harvesting the radius from rats, wrapping both ends of each bone with a laboratory film leaving only the central 10 mm exposed, and demineralizing the central part by immersing the bone in 1.75% HCl solution. In the grafts prepared, the central part of the bone became semi-translucent and flexible while the ends remained as hard bone tissue, thus forming a B-T-B type graft. The tensile strength of the grafts was greater than that of the medial collateral ligaments of the rats and about the same as of their anterior cruciate ligaments. No inflammation or other adverse reaction was noticed in experimental subcutaneous transplantation and the grafts showed excellent biocompatibility. In experimental ligament reconstruction, the test animals did not show any impairment on gait. There was invasion of fibroblasts into the graft at 4 weeks, and the fibroblasts were found through the whole graft and what looked like ligament tissue could be seen macroscopically at 8 weeks. Besides, bone tissue had infiltrated into the inside cavity of the non-demineralized part of the graft and proliferation, resulting in good bone union.

The results obtained suggest that the grafts prepared in this study have a sufficient potential as B-T-B type grafts for ligament reconstruction.

Key words: ligament reconstruction, graft tissue, demineralized bone, collagen matrix, B-T-B type grafts

Introduction

Autologous transplantation of tendons and ligaments such as the patellar tendon, hamstring tendons, or the iliotibial tract is the mainstay in the reconstruction of ligaments, typified by the reconstruction of the anterior cruciate ligament (ACL). Among these, the patellar tendon harvested with the patella attached to one end and a piece of the tibia to the other end has the bone-tendon-bone (B-T-B) configuration. This type of graft is often used because it has various advantages. For example, it is superior in fixing strength because the bone portions at the ends can be fixed with interference fit screws and the like. Another advantage is that the length of the transplanted tendon after fixation of the ends of the graft is less, for instance, than when a hamstring tendon is used with a button and suture or post fixation and can therefore compensate for the low stiffness of the transplanted ligament. However, autologous transplantation using B-T-B patellar tendons with attached bone pieces has various unsolved problems like the delayed recovery of the strength of the quadriceps muscle, pain in the anterior part of the knee, reduced strength of the patellar tendon at the site of the harvest, and long operation time needed for harvesting the piece to be transplanted.

Apart from these autografts, it would be very useful if allografts could be produced from donors and used, particularly if B-T-B type grafts could be mass-produced from bone tissue.
Jackson D et al. had claimed good results in their experiments with goats where a collagen matrix prepared by demineralizing bone tissue with acid had been used as a graft for ligament reconstruction. However, their grafts were ligament alone type grafts similar to hamstring tendons or iliotibial tracts and cannot be considered as substitutes for B-T-B grafts.

In my study, the central part of a long bone was demineralized leaving the ends as non-demineralized bone tissue. This is a new method of preparing B-T-B type grafts similar to patellar tendons with bits of bone attached at both ends. I investigated whether the grafts could be used in ligament reconstruction, as a substitute for the patellar tendons with attached bits of bone.

Materials and Methods

All procedures performed on the animals were in accordance with the institutional guidelines for animal care at Tokyo Medical and Dental University.

Test animals

Thirty-five male SD rats, all about 12 weeks old, were used. They weighed 350-420 g each.

Design of experiment

Four types of experiments were conducted. These were the preparation of the grafts, tensile testing, experimental subcutaneous transplantation, and experimental ligament reconstruction.

Grafts were prepared from 28 radius of euthanized donor SD rats aged 12-16 weeks. 10 grafts were used for tensile testing, 8 for subcutaneous transplantation and 10 for ligament reconstruction.

Ten ACLs and 5 medial collateral ligaments (MCLs) taken from donor rats were used as controls in the tensile testing.

In the experimental subcutaneous transplantation, 2 grafts each were transplanted subcutaneously on the backs of 6 rats and 2 rats each were euthanized after 2, 4 and 8 weeks, for observations.

In the ligament reconstruction trial, MCL reconstruction surgery was carried out on the left knee joints of 10 rats. Histological observations were made on 5 euthanized rats each at 4 and 8 weeks after the transplantation surgery.

Preparation of grafts

Radius that had been harvested from donors and kept frozen at $-30^\circ$C were thawed and removed soft tissues thoroughly. Myeloid tissue was removed by drilling from both ends. The bones were then defatted by immersion in 95% ethanol (Kanto Chemical, Tokyo, Japan) for 6 hours followed by 3 hours immersion in 99.5% diethyl ether (Kanto Chemical, Tokyo, Japan), both at room temperature. The laboratory film (Parafilm®), American National Can, Wisconsin, USA) was wrapped around the ends of each bone leaving about 10 mm of the central region exposed. The bones were then immersed in 1.75% hydrochloric acid (HCl) solution which was diluted 35% hydrochloric acid (Kanto Chemical, Tokyo, Japan) in twenty times, being continuously stirred by a stirrer at $20^\circ$C (Fig. 1). They were taken out from the HCl solution when the central portion became semi-translucent and flexible because of demineralization. This change was observed in about 45 minutes. The laboratory film was then removed and the specimens washed thoroughly in distilled water to remove the HCl solution. They were stored at $-30^\circ$C until used in the following experiments.

The partially demineralized bone grafts had the hard portion at their two ends and the central demineralized portion had a semi-translucent appearance with sufficient flexibility (Fig. 2 a,b).

Tensile test

Ten of the prepared grafts were subjected to tensile testing. Because the grafts were of the B-T-B type, two holes of diameter 0.25 mm were made at the non-demineralized parts at each end. Zero point two mm diameter stainless steel wires were passed through these holes and tied to make anti-slippage anchors. The non-
demineralized ends were embedded in self-curing acrylic resin (Ostron®/C100, GC Dental Industrial Corp., Tokyo, Japan). The specimen was then clamped in a tensile tester (Instron®1132, Instron, Massachusetts, USA) and tested at the testing speed 20 mm/min. The maximum failure load (MFL) and linear stiffness (LS) were obtained from the results of the tensile testing.

For comparison, 10 knee joint specimens with ACL and 5 knee joint specimens with MCL were used as control specimens in the tensile testing. These were harvested from rats of the same strain with entire femur and tibia attached. All soft tissues other than the ACL or MCL were removed from the specimens and they were secured with stainless steel wires and embedded with acrylic resin, as with the graft specimens, before testing.

Experimental subcutaneous transplantation

The grafts prepared above were transplanted subcutaneously on the back of rats. Ketamine hydrochloride (90 mg/kg, i.p.; Veterinary Ketalar 50®, Sankyo, Tokyo, Japan) and medetomidine (0.5 mg/kg, i.p.; Domitor® Meiji Seika, Tokyo, Japan) were administered intraperitoneally for general anaesthesia of the animal. Grafts were disinfected in the 70% ethanol for 5 minutes and washed thoroughly in sterilized physiological saline solution, just before transplantations. Subcutaneous incisions were made on the back of the animal and two grafts were transplanted per animal, one on the left side and another on the right side, and fixed by sutures to the fascia of the back muscles. The incisions were then stitched up. After the surgery, atipamezole (1 mg/kg, i.p.; Antisedan® Meiji seika, Tokyo, Japan) antagonized the action of medetomidine was administered subcutaneously to speed up recovery from the anaesthesia. Enrofloxacin (10 mg/kg, s.c.; Baytril®, Bayer-Japan, Tokyo, Japan) was also administered subcutaneously to prevent infection.

Next, a tunnel was made in the tibia diagonally downwards from the attachment site of the MCL towards the lateral direction. As in the femur, two small holes were made and the nylon suture ends were led into the small holes and pulled until the distal non-demineralized part of the graft was pulled into the tunnel, and the sutures ligated giving suitable tension to the graft (Fig. 3a). The view immediately after transplantation of the graft are shown in Fig. 3b. The skin incision was then sutured by a standard method. After the surgery, atipamezole (1 mg/kg, i.p.) was administered subcutaneously and enrofloxacin (10 mg/kg, s.c.) was also administered to prevent infection.

The rats were euthanized 4 or 8 weeks after the transplantation and knee joint removed for macro and microscopic observations. The femur and tibia were cut at mid-length for removing the graft-transplanted knee joint. The harvested joints were fixed for 1 week in 10% buffered formalin (Oriental Pharmaceutocal, Yamagata, Japan) and then demineralized for the preparation of the tissue specimen stained with hematoxylin and eosin.

**Experimental ligament reconstruction**

Ketamine hydrochloride (90 mg/kg, i.p.) and medetomidine (0.5 mg/kg, i.p.) were administered intraperitoneally to the experimental rats for general anaesthesia. Body hair was cut off from around the left knee joint and the area was sterilized with 70% isopropyl alcohol (Maruisi Pharmaceutical, Tokyo, Japan) and povidone iodine (Veterinary Isodine, Meiji Seika, Tokyo, Japan). Grafts were disinfected in the 70% ethanol solution for 5 minutes and washed thoroughly in sterilized physiological saline solution, just before transplantations.

The skin on the medial side of the left knee joint was incised. The MCL was cut at the sites of attachment to bone, and removed. A tunnel (diameter 2.5 mm) was drilled in the femur from the attachment site of the MCL, diagonally towards the upper lateral direction. However tunneling was stopped before the cortical bone on the lateral side. Two small holes were made from the tip of the tunnel onwards using a Kirschner wire (diameter 0.8 mm). A 23G injection needle was inserted into one hole from the lateral towards the medial side and one end of the 4-0 monofilament surgical nylon sutures (diameter 0.15 mm) already fixed to the graft was led into the needle. The other end of the nylon suture was lead into the other hole in the same manner. The two nylon suture ends drawn out towards the lateral side were pulled, until the non-demineralized part of the graft entered the femur, and then ligated.

**Experimental subcutaneous transplantation**

The grafts prepared above were transplanted subcutaneously on the back of rats. Ketamine hydrochloride (90 mg/kg, i.p.; Veterinary Ketalar 50%, Sankyo, Tokyo, Japan) and medetomidine (0.5 mg/kg, i.p.; Domitor® Meiji Seika, Tokyo, Japan) were administered intraperitoneally for general anaesthesia of the animal. Grafts were disinfected in the 70% ethanol for 5 minutes and washed thoroughly in sterilized physiological saline solution, just before transplantations. Subcutaneous incisions were made on the back of the animal and two grafts were transplanted per animal, one on the left side and another on the right side, and fixed by sutures to the fascia of the back muscles. The incisions were then stitched up. After the surgery, atipamezole (1 mg/kg, i.p.; Antisedan® Meiji seika, Tokyo, Japan) antagonized the action of medetomidine was administered subcutaneously to speed up recovery from the anaesthesia. Enrofloxacin (10 mg/kg, s.c.; Baytril®, Bayer-Japan, Tokyo, Japan) was also administered subcutaneously to prevent infection. The animals were euthanized 2, 4 or 8 weeks after the surgery to observe the condition of the transplanted grafts.
Results

Tensile test

All the graft specimens ruptured at the demineralized central zone.

Representative Load-elongation curves of the grafts and the controls are shown in Fig. 4.

The maximum failure load of the grafts was 33.1±4.0N (mean±SD, expressed in this format hereinafter). The linear stiffness was 31.1±4.5 N/mm (Table 1).

In 3 out of the 10 control ACL specimens, epiphysiodesis occurred before ligament failure. These three cases were not included in the calculation of the mechanical properties. The maximum failure load of the ACLs was 35.2±3.2N and the linear stiffness was 27.0±6.3N/mm (Table 2). These were 11.6±4.9N and 2.0±0.9N/mm respectively for the MCL (Table 3).

Experimental subcutaneous transplantation

Two weeks after the subcutaneous transplantation, grafts remained intact as such in all the observed cases although they were covered with granulation tissue having high penetration of blood vessels from the skin and the fascia (Fig. 5a). Four weeks after transplantation, grafts remained more or less as such in all the

Table 1. The maximum failure load (MFL) and linear stiffness (LS) of the grafts. SD: Standard deviation.

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Table 2. Maximum failure load (MFL) and linear stiffness (LS) of ACLs of the rats. SD: Standard deviation.

<table>
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Fig. 3. Experimental reconstruction of the medial collateral ligament of rat. Tunnels were made in the femur and tibia, the graft transplanted there and fixed with nylon sutures (diameter 0.15 mm) (a). The view immediately after transplantation of the graft. The graft was fixed with a suitable tension (b).

Fig. 4. Representative Load-elongation curves of the graft, ACL and MCL of rat. Linear stiffness is obtained dividing maximum failure load value with elongation value at maximum failure load.

This indicator shows the point of maximum failure load of each graft.

Fig. 5. Experimental reconstruction of the medial collateral ligament of rat. Tunnels were made in the femur and tibia, the graft transplanted there and fixed with nylon sutures (diameter 0.15 mm) (a). The view immediately after transplantation of the graft. The graft was fixed with a suitable tension (b).
observed subjects, but, compared to the conditions observed at 2 weeks, there was less coverage by granulation tissue, and the specimens had decomposed slightly and were observed as a vulnerable tissue (Fig. 5b).

Eight weeks after the transplantation, the demineralized part was almost completely absorbed while the non-demineralized parts remained (Fig. 5c).

There was no sign of inflammation in any of the subjects observed at 2, 4 or 8 weeks.

Experimental ligament reconstruction:

All the test animals with reconstructed MCL of the knee joint did not show any impairment on gait 4 and 8 weeks after surgery. When observed 4 weeks after the transplantation, granulation was seen around the graft, although not much of the graft was covered. After removing the surrounding tissue, the transplanted graft was seen to remain as such, with little sign of absorption having taken place (Fig. 6a,b). Fibroblasts had infiltrated into the graft and grown there (Fig. 6c). The bone tissue had penetrated from the surroundings into the inside cavity of the non-demineralized part at the two ends, showing ossification (Fig. 6d).

At 8 weeks, the macroscopic appearance of the graft had been transformed into white ligament-like tissue.

Table 3. Maximum failure load (MFL) and linear stiffness (LS) of MCLs of the rats. SD: Standard deviation.

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<td>mcl3</td>
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<tr>
<td>Average</td>
<td>11.6</td>
</tr>
<tr>
<td>SD</td>
<td>4.9</td>
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</tbody>
</table>

Fig. 5. A graft after 2 weeks transplantation. Grafts remained intact although they were covered with dense granulation tissue (a). A graft after 4 weeks transplantation. This specimen was looked vulnerable (b). A graft in the subcutaneous tissue after 8 weeks transplantation. The demineralized part was almost completely absorbed while the non-demineralized parts remained (c).

Fig. 6. Four weeks after transplantation (a-d). In the macroscopic observation, the graft was seen as a whitish tissue (a). In the microscopic observation, the transplanted graft was seen to remain without any adverse reaction such as inflammation (b). Fibroblasts had infiltrated and grown in the part corresponding to the ligament both on the surface and inside of the cavity of the graft (c). The bony part of the graft remains within the tunnel in the bone and bone tissue has infiltrated into the cavity of the graft, demonstrating bony union (d).

►: This indicator point out the graft. t: tibia. f: femur. bg: bony part of graft. ca: the cavity of the non-demineralized part of the graft.
Fig. 7. Eight weeks after transplantation (a-d). In macroscopic observation, the transplanted graft was seen transformed into fibrous ligament tissue (a). Histological observation showed that the graft had become fibrous tissue (b). A magnified view of the graft at the part corresponding to the ligament showed proliferation of fibroblasts in the graft (c). The non-demineralized part at the end of the graft had fused with surrounding bone tissue. Proliferation of chondrocytes was also seen in some areas (d).

Microscopic examination also showed that fibroblasts had proliferated and that the graft was in the process of being converted into ligament-like tissue (Fig. 7b,c). The non-demineralized bone at the ends had fused with the surrounding bone tissue and chondrocyte proliferation was also observed in some parts (Fig. 7d).

Discussion

Bone tissue consists of about 65% of inorganic component and the main constituent of that is hydroxyapatite. Thirty-five% is organic component of which 97% is type I collagen. The remainder consists of proteoglycans, glycoproteins, related biomacromolecules, etc. Contrary to this, the main constituents of ligament tissue are water and organic components and type I collagen accounts for 70% of the organic components. Therefore, I assumed that if the bone was demineralized to remove the inorganic components, it would acquire a composition similar to that of ligament, and consist mainly of type I collagen. Also, proteoglycans, glycoproteins and related biomacromolecules are known to induce biological reactions favourable for inward growth and differentiation of cells, and tissue reconstruction. So, I felt that I could expect these remaining components to promote ligament reconstruction. I therefore designed this experiments taking these biological advantages into account and evaluated the potential of these grafts in ligament reconstruction.

The central demineralized parts of the prepared grafts were semi-translucent. They retained the shape they had before the demineralization but were found to be flexible when force was applied from the side. It was therefore concluded that they had the mechanical properties suitable for a ligament reconstruction material.

In the tensile tests, the grafts showed far greater maximum failure load (MFL) than that of rat MCL, and the MFL was almost as high as that of ACLs. From this, it appeared that the grafts had the initial strength suitable for being used as reconstruction material for MCLs and ACLs.

The load-elongation curves of the prepared grafts were similar to those of ACLs used as controls. Besides, they resembled ACLs in linear stiffness also. Thus, it is likely that the mechanical properties of the grafts were close to those of ACLs.

No adverse reactions including inflammation were noticed in the experimental subcutaneous transplantation. This suggested that the demineralization treatment did not cause problems with regard to the biocompatibility of the material. The grafts remained as such for 2 and 4 weeks after the subcutaneous transplantation, although at 4 weeks there were some signs of the grafts getting decomposed and absorbed. At 8 weeks, grafts were almost completely absorbed. These findings are similar to the results obtained when transplanted ligaments and tendons had not been dynamically stimulated. In other words, the grafts prepared by us induce vital reactions similar to those induced by
ligament tissue.

Unlike in the experimental subcutaneous transplantation, in the experimental reconstruction of ligaments, there was little advancement of absorption at 2 and 4 weeks after transplantation and there was proliferation of fibroblasts. At 8 weeks, the graft was seen as a white fibrous ligament-like tissue. Histological observations showed further proliferation of fibroblasts, suggesting that the graft was gradually transforming into ligament tissue. These results suggest that when dynamic mechanical stimuli are applied to the graft in the living body, they not only maintain their function but also get maturated as ligament tissue. Besides, bone tissue was seen to penetrate from the surroundings into the non-demineralized part at the both ends of the graft, indicating ossification and strengthening of the bond between the graft and the bone. The formation of chondrocyte was also seen at the boundary between the part of the graft corresponding to the ligament and the bone. This suggested the possibility of the formation of a four-layer structure of bone - calcified cartilage - fibrous cartilage - ligament tissue, which is the actual structure at ligament attachment sites.

The radius, which is long hollow bone, was used for preparing the grafts in this study. So, the graft was hollow. This hollow part was connected to the medullary cavity of the femur and the tibia in the transplantation. This arrangement apparently allowed easy infiltration of cells from the marrow into the graft. In fact, proliferation of fibroblasts was observed in the graft from an early stage after transplantation. It is very likely that the hollow structure promoted infiltration of cells into the graft as well as acting as a scaffold, promoting speedy regeneration.

Until now, ligament reconstruction has been done mainly using autografts. Even in such autografts, the presence of live cells in the graft is not always necessary. The collagen fibres of the transplanted tendon provide the dynamic functions for a short while. In the long term, it is believed that cells from the surrounding tissues infiltrate into the graft and assist in the maintenance of the collagen fibres and their maturation as ligament tissue. In this study, no live cells were present in the prepared grafts before transplantation, because of the treatment given to the graft. Nevertheless, as the results of the experiments show, fibroblasts infiltrated into the grafts and multiplied there, helping to maintain the collagen fibres, demonstrating good regeneration. The grafts had satisfactory mechanical properties such as initial strength, flexibili-

ty and also had other characteristics like biocompatibility and ability to induce formation of new ligament tissue.

On the basis of the above results, it was suggested that the bone-demineralized bone-bone graft prepared in the present study by partial demineralization of the bone has a sufficient potential for use as a substitute for B-T-B grafts in ligament reconstruction.

In this study, grafts were disinfected in 70% ethanol solution, but I don’t think it is sufficient for clinical application. It is necessary to perform ethylene oxide or gamma irradiation sterilization after freeze-drying grafts and to re-inspect biomechanical property of them. Furthermore, the biomechanical tests of regenerated MCLs were not carried out. I plan to undertake further histological and biomechanical investigations of them.

Acknowledgements

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