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Bleeding from the Bone Marrow Enhances Remodeling of the In Situ Frozen-thawed Anterior Cruciate Ligament

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Abstract

Introduction. The purpose of this study is to biomechanically and histologically evaluate the effect of bleeding from bone marrow on remodeling of the in situ frozen-thawed anterior cruciate ligament.

Methods. Forty-four rabbits were used. Eight rabbits were used to evaluate the amount of bleeding at days 1-4. Thirty-six rabbits were divided into 2 groups, after the right anterior cruciate ligament underwent the freeze-thaw treatment. In Group I, no treatments were applied. In Group II, a tunnel was drilled into the bone marrow the femoral intercondylar notch. Each rabbit was sacrificed at 6 or 12 weeks. We examined the mechanical properties, and the histology of the anterior cruciate ligament.

Results. A small amount of blood clot was observed only on the first day in Group I, while a large amount of blood clot was seen around the anterior cruciate ligament for 3 days after surgery in Group II. In the midsubstance, a number of cells were scattered in Group II, no cells were seen in Group I at 6 weeks. The tangent modulus showed some tendency of difference between Groups I and II at each period.

Discussion. Bleeding from the bone marrow obviously enhanced extrinsic cell infiltration into the in situ frozen-thawed anterior cruciate ligament at 6 weeks, and

showed some effects on its mechanical properties. This study implied that, in anterior cruciate ligament reconstruction, the blood from a bone tunnel play an important role in graft remodeling.

1. Introduction

After anterior cruciate ligament (ACL) reconstruction, intrinsic fibroblasts in the tendon graft are necrotized, and then, extrinsic cell infiltration with revascularization occurs in the graft (Arnoczky et al., 1982; Jackson et al., 1993). The mechanical properties of tendon autografts are reduced once after surgery, and then, gradually improve, although the properties remain abnormal even at 12 months after surgery (Butler et al. 1989, Jackson et al., 1993). On the other hand, Arnoczky et al. (1982) reported that, immediately after ACL reconstruction, the intercondylar space around the grafted tendon is filled with blood clot mainly due to bleeding from the bone marrow. It is known that blood clot promotes tissue repair (Anitua et al., 2004; Bab and Einhorn, 1994). Therefore, there is a strong possibility that the blood clot due to bleeding from the bone marrow may significantly affect the remodeling of the tendon graft after ACL reconstruction. However, no studies have evaluated the effect of the blood clot due to bleeding from the bone marrow on the tendon graft, because it is extremely difficult to distinguish the effect of the blood clot from effects of other factors in common ACL reconstruction models, in which a free tendon graft is transplanted into bone tunnels.

Recently, the in situ frozen-thawed ACL, which are anatomical but acellular,

have been used as an idealized ACL reconstruction model for many biomechanical studies (Jackson et al., 1991; Katsuragi et al., 2000), and has been used to determine the effect of a local application of various growth factors (Azuma et al., 2003; Ju et al., 2006; Nagumo et al., 2005; Sakai et al., 2002). In this model, the joint cavity is filled with a small amount of blood clot from peripheral vessels, but bleeding from bone marrow does not occur at all, because no bone tunnels are created. Therefore, by using this model, the effect of blood clot from bone marrow on the frozen-thawed ACL can be distinguished from effects of other factors. In the present study, we have hypothesized that bleeding from the bone marrow may significantly affect the mechanical properties and histological profiles of the in situ frozen-thawed ACL. The purpose of this study is to test this hypothesis.

2. Methods

2.1. Study design

This study was performed with 44 mature female Japanese White rabbits weighing 3.4 (0.3) kg (mean (standard deviation)). Animal experiments were carried out in the Institute of Animal Experimentation, Hokkaido University School of Medicine under the Rules and Regulation of the Animal Care and Use Committee,

Hokkaido University School of Medicine. Thirty-six out of 44 rabbits underwent the in situ freeze-thaw treatment described below in the right ACL to kill intrinsic fibroblasts. Then, they were randomly divided into two groups, Groups I (n=18) and II (n=18). In Group I, we did not apply any additional treatments. In Group II, a tunnel having a diameter of 2 mm and a length of 40 mm was drilled at the center of the roof of the femoral intercondylar notch in the right knee to fill the knee joint with blood from the bone marrow, because this treatment mimicked ACL reconstruction. In previous studies (Ballok et al., 1989; Yoshikawa et al., 2006), approximately 2-3 mm tibial and femoral drill holes were made to place the patella tendon graft similar to the normal ACL in terms of the cross-sectional area. According to these studies, we chose the 2-mm hole. In addition, the length of the femoral tunnel created the lateral condyle in the rabbit is commonly 40-50 mm. Therefore, we chose 40-mm tunnel in this study. The left knee of the 36 rabbits in Groups I and II was an unoperated control. No animals were immobilized postoperatively, and all were allowed unrestricted daily activities in their cages (52 cm in width, 35 cm in height, and 33 cm in depth). Nine rabbits in each group were sacrificed by a lethal injection of pentobarbital at 6 and 12 weeks after the treatment, respectively. At each period, 7 and 2 of the 9 rabbits were used for tensile tests to determine the mechanical properties and histology of the ACL,

respectively. Nine knees were randomly harvested from all the left knees, and the same examinations as performed for the treated ACL specimens were carried out to obtain contra lateral control data.

To observe the intraarticular blood clot formation in the early phase after the drilling treatment, the remaining 8 rabbits were used. After the freeze-thaw treatments were applied to the bilateral ACL in each animal, a tunnel having a diameter of 2 mm and a length of 40 mm was drilled at the center of the roof of the femoral intercondylar notch in the right knee and no additional treatment was applied in the left knee. Two rabbits were killed by a lethal injection of pentobarbital at 1, 2, 3, and 4 days after treatment, and blood clot formation in the joint cavity were macroscopically observed in both the knees.

2.2. Surgical procedure and postoperative treatment

Surgery was performed under anesthesia (intravenous injection of pentobarbital, 25 mg/kg). A midline longitudinal skin incision was made in the right knee and the ACL was exposed through a medial parapatellar approach. We applied the freeze-thaw treatment to the ACL three times using the previously developed cryo-probe (Katsuragi et al., 2000) and the below-described technique to kill the

fibroblasts of the ACL (Fig. 1). This probe was composed of a thin stainless-steel tube and a urethane-foam coated plastic syringe from which liquid nitrogen could be squeezed into the tube. The probe was placed so as to envelop the ACL, and the ACL was frozen by squeezing liquid nitrogen into the tube, and then thawed by pouring saline solution of 25 degrees Celsius into the intraarticular space. This freeze-thaw technique was repeated three times for each ACL. A previous study in our laboratory revealed that 95 % of the cells collected from the femoral attachment portion of the ACL and 100 % of the cells from the mid-section and tibial attachment portion are killed in this model (Katsuragi et al., 2000). In Group II, a Kirschner wire having a diameter of 2 mm was drilled at the center of the roof of the femoral intercondylar notch in the right knee so that a tunnel having a length of 40 mm was created.

2.3. Sample preparation for mechanical testing

Both hindlimbs were resected immediately after sacrifice. Each hindlimb for mechanical testing was wrapped in gauze moistened with physiologic saline solution and then wrapped in air-tight plastic films. The specimens were stored at –32 degrees Celsius until testing. Before mechanical testing, each knee was thawed overnight at 4 degrees Celsius. The knee was removed from the hindlimb, and the surrounding

muscles were carefully dissected off.

2.4. Cross-sectional area of the ACL

The cross-sectional area of each ACL specimen was measured by an optical noncontact method using a video dimension analyzer (HTV-C1170; Hamamatsu Photonics, Tokyo, Japan) (Yamamoto et al., 1999). Briefly, a medial femoral condyle and an anterior portion of the lateral femoral condyle anterior to the ACL insertion were resected for visualization with the video dimension analyzer. The femur was attached to the stepping motor, and a constant tensile load of 0.5 N was applied to the ACL by suspending a weight from the tibia. The femur was rotated with the stepping motor at 5 degrees angular increments through 180 degrees, and the corresponding profile width of the ACL was recorded with the video dimension analyzer. The cross-sectional shape of the ACL was reconstructed using a computer algorithm (Yamamoto et al., 1999). The measurement was done at the middle of the ACL.

2.5. Mechanical properties of the anteromedial bundle of the ACL

To determine the mechanical properties of the ligament substance, we used only the anteromedial bundle of the ACL for tensile testing (Woo et al., 1992; Sakai et

al., 2003; Azuma et al., 2003; Nagumo et al., 2005; Ju et al., 2006), because pilot tests showed that the whole ACL substance was stronger than the bone at the insertion sites. After measurement of the cross-sectional area of the whole ACL, the posterolateral bundle of the ACL was resected using a stainless-steel razor blade. The femur-ACL-tibia complex (FATC) with the remaining anteromedial bundle of the ACL was used for tensile testing. The cross-sectional area of the middle of the anteromedial bundle was measured again in the same manner. Then, the femur and the tibia were separately cast in cylindrical aluminum tubes (25 mm diameter and 30 mm length) using polymethylmethacrylate resin. For tensile testing, the femur-anteromedial bundle-tibia complex specimen was mounted onto a tensile tester (PTM-250W, Orientec, Tokyo, Japan) with the use of a set of specially designed grips (Fig. 2). Then, the tibia was flexed at 45 degrees to the femur. The tibia was rotated approximately 90 degrees toward the internal direction against the femur to remove the normal distortion of the anteromedial bundle so that all portions of the bundle were uniformly loaded during tensile testing (Woo et al. 1992). Two parallel lines were drawn transversely on the surface using Nigrosine stain as gauge-length markers for strain measurement. The distance between the two lines was approximately 5 mm. Prior to the tensile test, the specimen was preconditioned with a static preload of 0.5 N for 5 minutes, followed by

ten cycles of loading and unloading (3% strain) with a crosshead speed of 5 mm/min. Then, each specimen was stretched to failure at a crosshead speed of 20 mm/min. Strain in the ligament substance was determined with a video dimension analyzer using the previously described gauge length markers (Fig. 2).

2.6. Histological observations

In each limb intended for histological observation, the FATC was resected and fixed in a buffered 10 % formalin solution, decalcified, and cast in paraffin blocks. The sectioned specimens were stained with hematoxylin and eosin. Cellularity and shape of the nucleus of cells in the ligament substance were examined with light microscopy. For quantification of cellularity, ten regions of identical size were randomly chosen for each specimen. An average of the overall cell count was calculated and normalized by area (mm^2).

2.7. Statistical analysis

All data were shown as the mean and standard deviation values. Concerning each parameter, the analysis of variance (ANOVA) was performed among the groups at each period. When a significant effect was obtained, post hoc tests with Fisher's

protected least significant difference test were made for multiple comparisons. Significance level was set at $p=0.05$.

3. Results

3.1. Observation of the blood clot formation in the early phase after surgery

In gross observation, the right knee with the drilling treatment was filled with much blood clot for 3 days after the treatment (Fig. 3-a), while the blood clot started to disappear from the knee on the fourth day. In the left knees without the drilling treatment, blood clot was not obviously observed on the second day, although small amount of blood clot was seen only on the first day (Fig. 3-b).

3.2. Biomechanical evaluations

Concerning the cross-sectional area of the whole ACL, there were no significant differences in the cross-sectional area of the whole ACL between Groups I and II at each period, while the area of the two groups was significantly greater than that of the normal control at each period ($p<0.0007$) (Table 1).

The average stress-strain curves are shown in Fig. 4. In tensile testing of the anteromedial bundle of the ACL, failure modes were shown in Table 2. Five

specimens in each group were torn in the midsubstance of the bundle at 6 weeks. Six and seven specimens in Groups I and II, respectively, were torn in the midsubstance of the bundle at 12 weeks. Only the specimens torn in the midsubstance of the bundle were used to determine the mechanical properties of the anteromedial bundle.

Concerning the tangent modulus, there were no significant differences between Groups I and II at each period. The tangent modulus of the two groups was significantly lower than that of the normal control at 6 weeks ($p<0.0210$). At 12 weeks, no significant difference was found between Group II and the control (Table 1), while the tangent modulus of Group I was significantly lower than that of the normal control ($p=0.0039$). Regarding the tensile strength, there were no significant differences between Groups I and II at each period, while the tensile strength of the two groups was significantly lower ($p<0.0047$) than that of the normal control (Table 1). No significant differences occurred in the strain at failure among the groups at each period (Table 1).

3.3. Histological observations

There were distinct differences in the histology between the two groups at 6 weeks. The cell densities in Groups I and II were averaged 0 cells/mm^2 and 658

cells/mm^2, respectively. No cells were observed in the midsubstance of the ACL except in the peripheral margins in Group I at 6 weeks (Fig. 5-a), while many cells with a round or ovoid nucleus were scattered in the midsubstance except in the core portion in Group II (Fig. 5-b), although we could not make statistical comparisons. At 12 weeks, cells with a round or ovoid nucleus were scattered in the core portion of the ACL in Groups I and II (Fig. 5-c, d). Cell densities in Groups I and II were averaged 962 cells/mm^2 and 1030 cells/mm^2, respectively. We could not find any obvious differences among the groups.

4. Discussion

In this study, histologically, bleeding from the bone marrow obviously enhanced extrinsic cell infiltration into the in situ frozen-thawed ACL at 6 weeks. Biomechanically, the tangent modulus of the in situ frozen ACL at 12 weeks appeared to be greater in the hemarthrosis group, although it was not statistically significant. Thus, this study demonstrated that bleeding from the bone marrow significantly affected remodeling of the in situ frozen-thawed ACL.

In this study, we used the in situ frozen-thawed ACL, in order to distinguish the effect of blood clot from bone marrow from effects of other factors. In an animal studies,

several authors reported an ACL reconstruction models using free autografts. However, there is a huge difference between animal ACL reconstruction and human ACL reconstruction, including the mechanical properties of the ACL graft, size of the knee, weight bearing condition, and so on. In addition, surgical procedures are different concerning fixation devices, precise technique with arthroscopy, and useful guide systems, and etc. Finally, applying adequate rehabilitation is extremely difficult for animals. Therefore, in animal studies, graft failure or elongation more frequently occurs after surgery. Previous biomechanical studies (Ballock et al., 1989) reported that a significant joint instability might persist after ACL reconstruction. If we used such an animal ACL reconstruction model with a free graft, it is difficult to detect the effect of blood clot from effects of the above-described factors that lead to unsuccessful results. The advantages of the in situ frozen-thaw ACL reconstruction model are the maintenance of anatomic attachment, physiologic volume, physiologic fiber orientation, and physiologic surrounding environment. In this study, we did not create the sham-treated group for the freeze-thaw treatment, because the authors' previous study (Sakai et al., 2002) clarified the natural remodeling course of the in situ frozen-thawed rabbit ACL. Namely, the freeze-thaw treatment significantly decreased the tensile strength and the tangent modulus of the ACL at 12 weeks after treatment. In

addition, the freeze-thaw treatment significantly increased the cross-sectional area and the water content of the ACL at 12 weeks and increased the percentage of thin collagen fibrils in the ACL at the same period. These results are supported by Jackson's study using a goat model (Jackson et al., 1991). Therefore, the in situ frozen-thawed rabbit ACL model has been accepted as a useful model for biomechanical studies (Azuma et al., 2003; Ju et al., 2006; Nagumo et al., 2005). Therefore, the study design of the present study is acceptable to clarify the effect of blood from a bone tunnel on the in situ frozen-thawed ACL.

Concerning the repopulation of the graft with cells, in the non-hemarthrosis group, no cells were seen in the midsubstance at 6 weeks, while a number of cells were scattered at 12 weeks. This effect of time is consistent with previous work in this same model (Sakai et al., 2003). On the other hand, in the hemarthrosis group, many cells with a round or ovoid nucleus were scattered in the midsubstance at each period. These results implied that bleeding from the bone marrow obviously enhanced extrinsic cell infiltration into the in situ frozen-thawed ACL, and showed some effects on its mechanical properties.

We consider causes why does bleeding from the bone marrow significantly affect remodeling of the in situ frozen-thawed ACL. The blood clot from bone marrow

contains various types cells and various growth factors such as platelet-derived growth factor (PDGF), transforming growth factor (TGF)-beta, insulin-like growth factor, and osteogenic growth peptide (Bab and Einhorn, 1994). Therefore, an application of blood clot promotes tissue repair (Anitua et al., 2004; Bab and Einhorn, 1994). A series of the authors' studies (Azuma et al., 2003; Sakai et al., 2002; Nagumo et al., 2005) reported that intra-articular administration of TGF-beta induces the extrinsic cell infiltration into the in situ frozen ACL and prevents the deterioration of its mechanical properties. Therefore, we speculate that TGF-beta contained in the blood clot from the bone tunnel might the extrinsic cell infiltration into the in situ frozen ACL. However, the effect of bleeding from bone marrow on the mechanical properties was not obvious. A possible reason is that we might have removed some of the more mature or immature healing tissue from the whole ACL. This might potentially increase the variability in the mechanical properties. However, this resection was required to determine the mechanical properties of the ACL midsubstance, since midsubstance failure occurs rarely when the femur-whole ACL-tibia complex is tested (Woo et al., 1987). This is an additional limitation of this study. Regarding PDGF from main platelet alpha-granule content, Hildebrand et al. (1998) reported that the application of PDGF significantly enhanced healing of the medial collateral ligament injury in a

rabbit model. Recently, however, Nagumo et al. (2005) reported that PDGF has no effect on the mechanical deterioration of in situ frozen ACL. It is important to compare this study with previous studies. Ishizue et al. (1990) demonstrated the effects of hemarthrosis on the ACL in rabbit. They reported that the total collagen content, collagenase activity, and biomechanical properties of the ACL were unaltered. However, we could not directly compare to our study, because they used as native ACL model with peripheral blood. In addition, it is known that the blood clot from bone marrow contains various types' cells and various growth factors compared that from peripheral blood.

There are some limitations to this study. The first limitation is that we resected the posterolateral bundle to determine the mechanical properties of the midsubstance of the ACL. Therefore, we could not determine the effect of the blood from bone tunnel on the structural properties of the femur-ACL-tibia complex. The second limitation is that we did not measure contents in the blood and changes of the local concentration of the contents after surgery. The third limitation is that we could not make statistical comparisons in the histological findings, because of small sample size (n=2). Beyond these limitations, however, we believe that this study provides valuable information on the effect of blood from a bone tunnel on remodeling of the in situ frozen-thawed ACL.

In our series of previous studies (Sakai et al., 2003; Azuma et al., 2003; Nagumo et al., 2005; Ju et al., 2006), we measured the mechanical properties of the ACL harvested from the uninjured knee at 6 and 12 weeks in the same model and methods. These studies reported that there are no significant differences among the ACLs and the completely normal ACL harvested from healthy rabbits. Therefore, in the present study, we did not measure again the mechanical properties of the ACL harvested from the uninjured knees at 6 and 12 weeks. The anteromedial bundle of the ACL has some degree of physiological distortion. Woo et al. (1992) recommended to untwist the bundle specimen by applying the 90 degrees rotation to the tibia against the femur in order to allow for a more uniform stress distribution in the ligament tissue during the tensile testing. Namely, the mechanical properties of the bundle can be the most precisely determined in this manner. Therefore, in our previous studies (Sakai et al., 2002; Azuma et al., 2003; Nagumo et al., 2005; Ju et al., 2006) and in the present study, we used this method. In addition, Woo et al. (1992) described that the mechanical properties of the anteromedial and posterolateral bundles of the rabbit ACL. They reported that the femur-posterolateral bundle-tibia specimens were many avulsion failures in the failure mode than the femur-anteromedial bundle-tibia specimens. In tensile testing, therefore, our previous studies (Sakai et al., 2003; Azuma et al., 2003;

Nagumo et al. 2005; Ju et al., 2006) were used the anteromedial bundle of the rabbit ACL.

Our previous study (Ohno et al. 1996) demonstrated that biomechanical and histological changes in the patella tendon after in situ freezing. The tensile strength in the freeze group started to decrease by 3 weeks and continued to decrease thereafter, falling to 38% of the control value at 24 weeks. In this study, concerning the tangent modulus, there were no significant difference between the hemarthrosis group at 12 weeks and the control, although the hemarthrosis group at 6 weeks was significantly lower than the normal control. Therefore, the effects on remodeling may be discernible if the study was performed in the long term results, because the mechanical properties of in the non-hemarthrosis group may be deteriorated to more degrees at 24 weeks or more, compared to those at 12 weeks. To verify this speculation, we should conduct a long-terms following study.

This study demonstrated that the bleeding from the bone marrow enhances graft remodeling. It is important for surgeons to understand this fact as fundamental knowledge on ACL reconstruction. The present data itself can not change clinical practice immediately hereafter. However, when molecules in the blood clot from bone marrow that enhance graft remodeling will be clarified in the future, the molecules

may change our clinical practice. Therefore, further studies are needed to be conducted in the near future.

In conclusion, this study demonstrated that bleeding from the bone marrow obviously enhanced extrinsic cell infiltration into the in situ frozen-thawed ACL at 6 weeks, and showed some effects on its mechanical properties. This study implied that, in ACL reconstruction for patients, the blood from a bone tunnel plays an important role in graft remodeling.

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Legends of Figures

Figure 1: The procedure to freeze the ACL in situ. A specially designed cryoprobe, composed of a thin stainless-steel tube and a urethane-foam coated plastic syringe, was placed so as to envelop the ACL, and the ACL was frozen by squeezing liquid nitrogen into the tube. Nitrogen gas was ejected into the air.

Figure 2: Experimental apparatus for determining strain using a video dimension analyzer.

Figure 3: Gross observation of the knee joint: The knee with the drilling treatment was filled with much blood clot on the third day (a). In the knee without the drilling treatment, small amount of blood clot was seen only on the first day (b).

Figure 4: Stress-strain curves of the ACLs determined at 6 and 12 weeks (mean (SD)). A: Control and Groups I and II at 6weeks. B: Control and Groups I and II at 12weeks. The curve of the control group is drawn in each picture.

Figure 5: Histological observations of the midsubstance of the ACLs (A: Group I at 6 weeks; B: Group II at 6 weeks; C: Group I at 12 weeks; D: Group II at 12 weeks). The number of cells in the midsubstance at 6 weeks appears to be greater in Group II than in Group I. (Original magnification x50)

TABLE 1: Tissue dimensions and mechanical properties of the ACLs (mean (SD)).

	Period	Groups		*P-value*
		I	II	(Group I vs II)
Cross-sectional area of the whole ACL (mm^2) (Normal: 5.6 (0.8))	6 weeks	8.8(1.1)*	8.4 (0.9)*	*N.S*
	12 weeks	9.0(1.8)*	9.6(1.8)*	*N.S*
Tensile strength (MPa) (Normal: 75.9 (10.2))	6 weeks	57.5 (5.1)*	55.5 (8.8)*	*N.S*
	12 weeks	35.6 (13.9)*	47.7 (11.8)*	*N.S*
Tangent modulus (MPa) (Normal: 463.6(65.9))	6 weeks	350.2 (63.5)*	348.6(115.4)*	*N.S*
	12 weeks	253.6 (69.8)*	332.2(181.6)	*N.S*
Strain at failure (%) (Normal: 20.4 (5.8))	6 weeks	20.2 (5.6)	20.1 (8.2)	*N.S*
	12 weeks	15.0 (5.5)	18.9 (7.8)	*N.S*

Post-hoc multiple comparison using with Fisher's PLSD test: * $p<0.05$ (vs Normal)

TABLE 2: Failure modes of the femur-ACL-tibia complex.

		6 weeks		12 weeks	
Failure mode	Normal	I	II	I	II
Midsubstance	5	5	5	6	7
Avulsion	2	2	2	1	0