J. Biomed. Eng. Res.
Vol.26, No.2, 101-110, 2005

Osteogenic Potential of the Periosteum and Periosteal Augmentation for Bone-tunnel Healing

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(Received January 17, 2005. Accepted March 14, 2005)

Abstract: Periosteum and periosteum-derived progenitor cells have demonstrated the potential for stimulative applications in repairs of various musculoskeletal tissues. It has been found that the periosteum contains mesenchymal progenitor cells capable of differentiating into either osteoblasts or chondrocytes depending on the culture conditions. Anatomically, the periosteum is a heterogeneous multi-layered membrane, consisting of an inner cambium and an outer fibrous layer. The present study was designed to elucidate the cellular phenotypic characteristics of cambium and fibrous layer cells in vitro, and to assess whether structural integrity of the tendon in the bone tunnel can be improved by periosteal augmentation of the tendon-bone interface. It was found the cells from each layer showed distinct phenotypic characteristics in a primary monolayer culture system. Specifically, the cambium cells demonstrated higher osteogenic characteristics (higher alkaline phosphatase and osteocalcin levels), as compared to the fibrous cells. Also in vivo animal model showed that a periosteal augmentation of a tendon graft could enhance the structural integrity of the tendon-bone interface, when the periosteum is placed between the tendon and bone interface with the cambium layer facing toward the bone. These findings suggest that extra care needs to be taken in order to identify and maintain the intrinsic phenotypes of the heterogeneous cell types within the periosteum. This will improve our understanding of periosteum in applications for musculoskeletal tissue repairs and tissue engineering.

Key words: Osteogenic Potential, Bone Healing, Periosteum, Augmentation

INTRODUCTION

Periosteum has long been used as a source for mesenchymal cells in the application of tissue engineering for the repair of bone and cartilage defects [1]. Periosteum is responsible for the lateral growth of cortical bone [2] and has been known more for its osteogenic potential, as described by many investigators [3, 4] than for its chondrogenic potential. Fresh periosteum explants were used both in conjunction with the host bone to induce new bone formation [5-7] and in conjunction with a biodegradable polymer scaffold to tissue-engineer a long bone [8, 9]. Isolated periosteal cells were used to induce new bone formation in vivo [10-12] and also to tissue-engineer bone in a culture chamber [13].

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In addition to osteogenesis, chondrogenesis has been also observed with isolated periosteum or periosteum-derived cell transplants in various tissues [13-16], and autologous periosteal grafting was used to repair cartilage defects in both animal models and human [17-19]. However, periosteum-derived cells have a tendency to develop into hypertrophic chondrocytes with an expression of type X collagen [20], a tendency which is implicated in the ossification process of the territorial matrix [3].

Such multipotentiality of the periosteum in osteogenesis and chondrogenesis may be related to the heterogeneous characteristics of the periosteum. Anatomically, the periosteum is a heterogeneous multi-layered membrane, consisting of an outer fibrous layer and an inner cambium layer [21, 22]. While the outer fibrous layer of periosteum is composed of fibroblastic cells immersed in collagen fibers [22], the inner cambium layer consists of a variety of cellular types including osteo-progenitor cells, osteoblasts, and osteoclasts [23-25]. It was previously believed that the bone or cartilage formation mechanism was directly related to the proliferation of the cambium layer cells [21]. However, Ueno, et al. [26] demonstrated that

when the periosteum harvested from rabbit tibia was grafted into suprahyoid muscles, the fibrous layer cells differentiated into chondrocytes and formed cartilage tissue, which was later replaced by trabecular bone. In contrast, cambium layer cells appeared necrotic and subsequently disappeared.

To understand biological mechanisms regulating the osteogenic or chondrogenic potential of periosteum, many investigators have elevated periosteum patches from the bone surface, liberated cells from the isolated periosteum using enzymatic digestion methods, and expanded the population using a two dimensional culture environment [11, 14, 24, 27-30]. However, such enzymatic digestion methods of an isolated periosteum patch could result in two possible experimental artifacts. First, it is very likely that the harvest procedure of the periosteum patch from the bone could damage and lose the cambium layer [31]. Second, considering that the periosteum is a heterogeneous tissue, enzymatic digestion of the entire periosteum patch results in a mixed population of heterogeneous cells in the culture system. In fact, some investigators considered the periosteum as a homogeneous material. Nakahara, et al. [14] reported that harvested chick periosteum had homogeneous morphology with a sparsely dispersed cell population in collagenous fiber structures. Liberated cells from chick periosteum also showed homogeneous fibroblast-like morphology in monolayer cultures and possessed the potential to differentiate into osteoblasts or chondrocytes when inoculated in vivo. Although it is widely accepted that periosteum cells are multi-potential, cellular phenotypic characteristics and functional behaviors of fibrous and cambium layer cells are still not fully understood.

The objectives of the current study are to characterize heterogeneous cell population in the periosteum in a monolayer culture system, and as an application to tissue engineering to assess whether structural integrity of the tendon in the bone tunnel can be improved by periosteal augmentation of the tendon-bone interface. We hypothesized that the phenotypic characteristics of periosteal progenitor cells are distinctively specific to the fibrous and cambium layers. Furthermore, the study will test the hypotheses that the periosteal cambium layer has a stronger osteogenic potential than the periosteal fibrous layer, and that the periosteal augmentation of a tendon graft yields improved structural properties of the tendon-bone interface when the periosteal cambium side is in contact with bone than when the periosteal fibrous side is in contact with bone. The in vitro cellular phenotypic characteristics were evaluated in terms of the cell morphology and cell proliferation, and the production of alkaline phosphatase (ALP) and osteocalcin. For bone tunnel healing study, using a rabbit hallucis longus tendon (HLT) and calcaneus process model, the strength and integrity at tendon-bone interface under periosteal augmentation were evaluated biomechanically and histologically.

MATERIALS AND METHODS

Monolayer Culture of Periosteal Cells

Cell Isolation and culture

Fifty-four intact periosteum-bone blocks (about 5mmx10 mm) were harvested from the proximal medial area of the both right and left tibiae of twenty four 6-month-old New Zealand White (NZW) rabbits. After being completely cleaned of muscles and bone marrow, the periosteum-bone complexes were treated briefly with 0.25% trypsin for 30 minutes (Fig. 1-A). The samples were then digested with 0.2% Type II collagenase (17101-015, Invitrogen, CA, US) for a total of 240 minutes, consisting of two sequential 120-minute periods at 37°C in 5% CO_2 as follows. As the collagenase enzyme reacts first on the outer fibrous layer of the periosteum, most of the fibrous layer cells were liberated during the first 120-minute period of the collagenase digestion (Fig. 1-B). The remaining periosteum-bone complexes were further digested with collagenase enzyme for the second 120-minute period (Fig. 1-C). The periosteal cells released during this second digestion period were mostly cambium layer cells. Harvested fibrous and cambium layer cells were separately cultured in α-MEM culture medium, supplemented with 10% fetal bovine serum (FBS, 16000-036, Invitrogen, CA, US), 100nM dexamethasone (D-4902, Sigma, MO, US), and 50 μg/ml ascorbic acid (A-8960, Sigma, MO, US) at 37°C in 5% CO_2, and the culture medium was changed every other day. The cell morphologies were monitored under a phase contrast microscope.

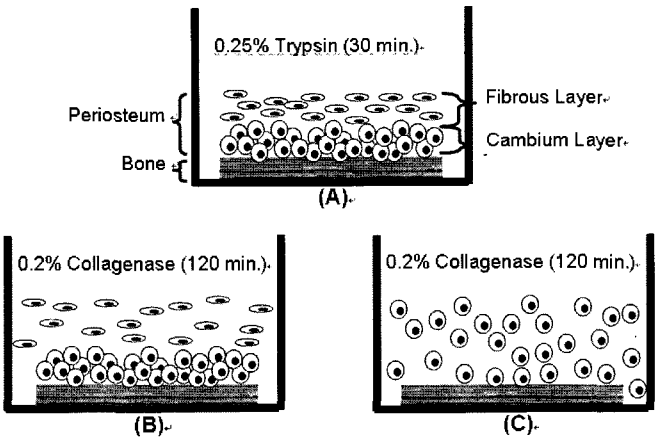


Fig. 1. Schematics of sequential enzymatic digestion method: (A) Intact bone-periosteum complex was first treated with 0.2% trypsin for 30 minutes. (B) During the first collagenase digestion (120 minutes), the majority of fibrous layer cells were liberated. (C) The remaining cambium layer cells were isolated during the second collagenase digestion (120 minutes).

Cell Proliferation Assay

To investigate the proliferation characteristics of the periosteal cells, the cells harvested from fibrous layer and cambium layer (n=6) were separately cultured in 6-well plates for 3 days, and the cell proliferation was evaluated using a 5-bromo-2'-deoxy-uridine (BrdU) assay kit (#1299964, Roche Molecular, CA, US).

Alkaline Phosphatase and Osteocalcin Assay

Alkaline phosphatase (ALP) production by the cultured fibrous and cambium cells were quantitatively measured using a commercial ALP assay kit (ALP-10, Sigma, MO, US) on days 0, 3, 6, and 9 of culture (n=8 per cell type per testing day). At the time of the assay, the cells were detached from the culture well with a trypsin-EDTA (25200-056, Invitrogen, CA, US), and the cell number was counted using a hemocytometer. A half million cells from each group were lysed by adding 500 μl of 1% (v/v) Triton X-100® (x-100, Sigma, MO, US) in PBS, and then underwent two cycles of freeze-thaw and sonication to dissociate the ALP enzyme from the cell membrane. The ALP content of the sample was then quantitatively represented in terms of the *p*-nitrophenol content per incubation time and normalized by the cell number (mol *p*-NP/min/cell-number).

The undercarboxylated osteocalcin (Glu-OC) production was quantitatively measured using a commercial enzyme immunoassay kit (model MK118, Takara Biomedicals Inc., Japan). Briefly, the fibrous and cambium layer cells (n=4 per cell type) were replenished with 2 ml of fresh culture medium on day 1, 4, and 7 of culture, and then the conditioned culture medium was harvested after a 48 hour incubation time (on days 3, 6, and 9 respectively).

Periosteal Augmentation for Bone-tunnel Healing

Animal Model and Surgical Procedures

Thirty male NZW rabbits weighing 2.5 to 3.0 kg (6-months-old) were used as an animal model, in which a tendon-bone tunnel healing model was created using the hallucis longus tendon (HLT) and the calcaneal posterior process of both hindlegs, similar to a model used by Liu et al. [32]. This model minimizes alterations of the biomechanics and load bearing in the ankle in rabbits, because the majority of the load in the hindlimb is supported by the Achilles tendon.

After both hindlegs were shaved, the medial frontal aspect of the proximal tibia was exposed via a 10-mm longitudinal incision of the skin. A 5 mm x 5 mm patch of periosteum was identified, the fibrous layer was marked with a surgical marker, and the periosteum patch was harvested using a surgical blade and a sharp elevator. Particular care was taken to minimize damage to the cambium layer of the periosteum patch. A 25-mm longitudinal incision then was made on the plantar aspect of the paw of the same leg and extended to the dorsal aspect of the Achilles tendon. The HLT then was identified and carefully separated from the dense plantar aponeurosis and subsequently transected at the distal end of the first metatarsal. Using a low-speed hand drill and a 2.38 mm (3/32 inch) diameter drill bit with continuous saline irrigation, a drill hole was made vertically at the middle of the calcaneal posterior process.

The portion of the HLT to be placed in the bone tunnel was first identified in the natural ankle position and then was treated with one of the four methods as described: Group A, periosteal graft was wrapped around the tendon with the fibrous layer facing toward the bone; Group B, periosteal graft was wrapped around the tendon with the cambium layer facing toward the bone; Group C, periosteal graft was subjected to three cycles of a freeze and thaw process to kill the cells, and then was wrapped around the tendon (positive control); and Group D, no periosteal graft was used with the tendon (negative control).

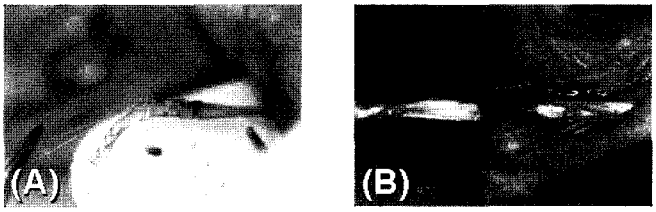


Fig. 2. (A) A rabbit's hallucis longus tendon was wrapped with a free periosteum patch. (B) A periosteum-wrapped hallucis longus tendon was passed through a 2.38-mm in diameter bone tunnel made in the calcaneus process.

In Groups A, B, and C, the periosteal graft was secured to the tendon using a 6-0 Vicryl suture (Fig. 2-A). To keep the diameter of the periosteally-augmented tendon graft similar to that of the negative group, the tendons in Groups A, B, and C were trimmed approximately 0.5 mm around the tendon before the periosteal augmentation. The periosteum-wrapped tendon was then passed through the drill hole (Fig. 2-B), and the distal end of the tendon was brought back around the bone and secured to the midsubstance of the tendon using an uninterrupted 3-0 proline suture. The tendon grafts in all groups had a snug fit within the bone tunnel. Finally, the incision was closed with 3-0 proline sutures and reinforced with staples.

The contralateral side also was operated on in the same manner except that each limb was assigned randomly to one of the four treatment groups so that

the 60 hindlegs from 30 rabbits were distributed equally among the groups (15 hindlegs per group). After surgery, all rabbits were returned to their normal cage activities ad libitum, and their daily behaviors were monitored closely. Animals were sacrificed at either 3 or 6 weeks after surgery, and the tendon-bone junctions were evaluated biomechanically and histologically as will be described. The animals used for the fluorescent histologic analyses received a series of fluorochrome treatments before sacrifice as will be described.

Biomechanical Testing

Twelve rabbits were sacrificed at 6 weeks after the surgery, and the tendon-to-bone interfaces from these animals (24 hindlegs; six from Group A, seven from Group B, six from Group C, and five from Group D) were evaluated biomechanically using a load-to-failure pull-out test immediately after sacrifice. The calcaneus-tendon specimen was separated from the experimental leg of the animal, and all other soft tissues were removed except the HLT and the calcaneous. The distal portion of the HLT wrapped around the bone and fixed to the midsubstance of the tendon was removed before the testing. The calcaneus bone then was fixed in a custom-built bone clamp, and the proximal free end of the tendon was fixed in a custom-built tendon clamp (Fig. 3).

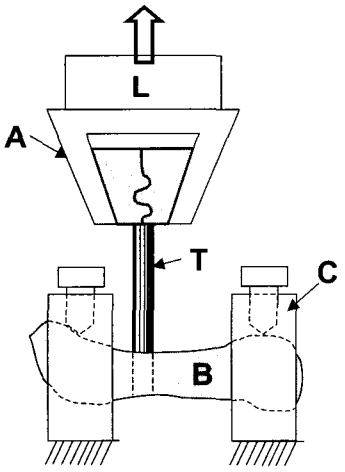


Fig. 3. Schematic of mechanical testing setup shows sinusoidal self-tightening tendon clamp (A), isolated calcaneus bone (B), calcaneus bone clamps (C), load-cell (L), hallucis longus tendon (T). The tendon was pulled at 10 inches per minute. The bone clamp consisted of two halo-rings and ten set screws, which allowed a flexible adjustment of the bone specimen in a horizontal plane. The tendon clamp consisted of a pair of interlocking sinusoidal teeth and wedges, which prevented the slippage of the tendon by a self-tightening mechanism.

The bone and tendon clamps were mounted in a material-testing machine (Instron® #1133, MA, US) with a 100 kg capacity tensile load-cell. After five cycles of preconditioning with 100 g, the tendon end was subjected to a load-to-failure pull-out test with a displacement rate of 10 inch per minute. All specimens were kept moisturized with spray of a fine mist of saline buffer solution throughout the entire experiment.

Histologic Analysis

To monitor the bone formation and remodeling process around the tendon in the bone tunnel, 10 of 30 rabbits (20 hindlegs, or five hindlegs per each group) received intramuscular injections of fluorochrome staining agents, 3% calcein (5 mg/kg, Sigma, MO, US) at Weeks 2 and 3, and 5% alizarin complexone (20 mg/kg, Sigma, MO, US) at Weeks 5 and 6, respectively. For the injection, all rabbits were sedated with a ketamine and xylazine cocktail (0.4 mL/kg), while fluorochrome agents were injected slowly to avoid causing adverse muscle, cardiac, and neural reactions. These rabbits were sacrificed 3 days after the final injection of alizarin complexone at the sixth week to allow a sufficient time for the injected fluorochrome to be fully incorporated into the specimen. After the animals were sacrificed, the calcaneus-tendon specimens were isolated from the rabbit legs and placed in a 4% formaldehyde solution at 4°C for 2 days. After gradual dehydration using ethyl alcohol and acetone for 2 hours, the specimen was embedded in methylmethacrylate, sectioned at 0.8 mm in thickness, and subsequently ground to approximately 50 μm thickness. The sections were stained with toluidine blue and alizarin red (5%), and examined under a light microscope. Also, newly formed bone structure around a bone-tunnel was identified by the appearance of calcein and alizarin complexone under a fluorescent microscope.

Eight additional rabbits had the same surgical protocol, were sacrificed at either 3 or 6 weeks after the surgery, and were used for histologic analyses with standard H&E. Two hindlegs were assigned to each period (3 or 6 weeks) per each surgical group, as described above. Two rabbits (3 and 6 weeks for Group C) were excluded from the study because of complications after the surgery.

Statistical Analyses

A one-factor ANOVA was used to analyze the effect of the treatment group on the load-to-failure test. Subsequently, a Fisher's protected least squares difference post-hoc test was used to examine differences between groups. All statistics were done

using Statview (SAS Institute, NC, US) at a significance level of 0.05.

RESULTS

Monolayer Culture of Periosteal Cells

Cell Harvest, Cell Morphology, and Cell Proliferation

The new sequential enzymatic digestion method effectively separated the fibrous and cambium layer cells. Figure 4-A is a histology picture of periosteum-bone complex after 30 minutes of trypsin treatment. After the collagenase digestion for the first 120-minute period, only cambium layer cells remained on the cortical bone (Fig. 4-B). The majority of the cambium layer cells were liberated during the second 120-minute period of the collagenase digestion. When the cells were seeded in a monolayer cell culture system, the cell morphologies of the fibrous layer cells and the cambium layer cells were distinct. The fibrous layer cells had a fibroblastic spindle shape and showed similar cell size (Fig. 5-A). In contrast, the cambium layer cells had a polygonal morphology and varying cell size (Fig. 5-B).

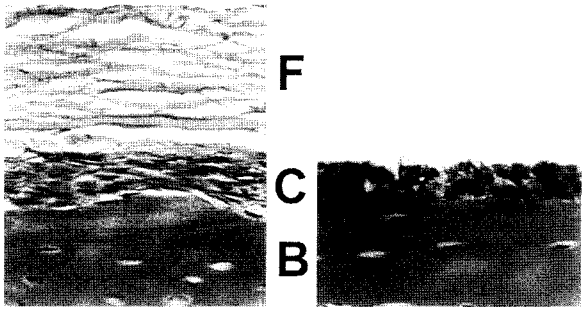


Fig. 4. Histology of a periosteum-bone complex during the sequential enzymatic digestion method. (A) Periosteum-bone complex after 30 minutes of the trypsin digestion, (B) After 120 minutes of the collagenase digestion. Only cambium layer cells were remained on the cortical bone. (F: fibrous layer, C: cambium layer, and B: Bone)

Fibrous and cambium layer cells proliferated at different rates. A large cell population growth was observed in the fibrous layer cells from Day 0 (Fig. 5-A) to Day 6 (Fig. 5-C). However, only a moderate increase of cell population was observed in the cambium layer cells from Day 0 (Fig. 5-B) to Day 6 (Fig. 5-D). This finding was validated with the BrdU cell proliferation assay. On Day 3, approximately 70 percent of fibrous layer cells showed positive BrdU activity (Fig. 6-A), whereas only 20 percent of cambium layer cells showed positive BrdU staining (Fig. 6-B). It was also observed that the majority of stained cambium layer cells were smaller than negatively stained cambium layer cells.

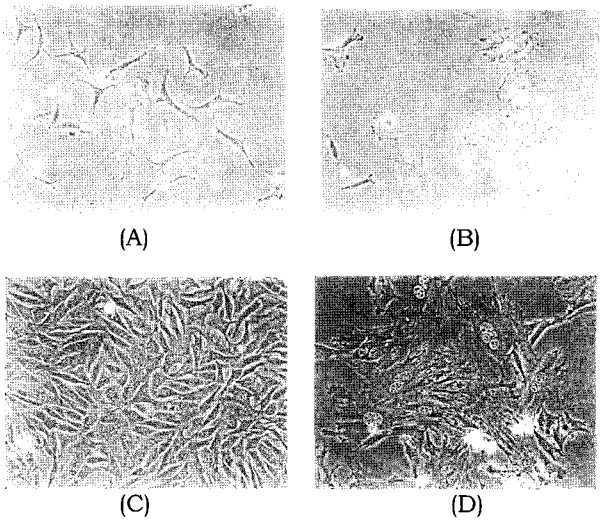


Fig. 5. Morphologies of the periosteal fibrous layer cells (A: day 3 and C: day 6) and cambium cells (B: day 3 and D: day 6) at x200.

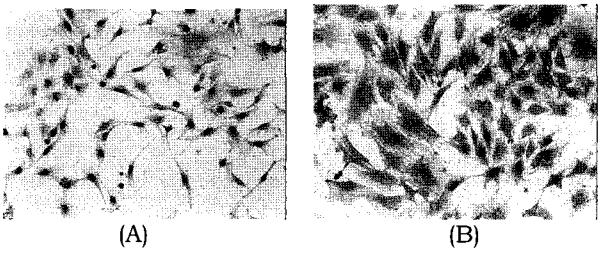


Fig. 6. Cell proliferation assay (BrdU). Fibrous layer cells (A: x200) and cambium layer cells (B: x400)

Alkaline Phosphatase and Osteocalcin Production

Expressions of ALP and osteocalcin are useful markers for osteoblasts and for the regulation of bone formation. Figure 7 shows that the cambium layer had significant ALP activity (represented by dark red staining), indicating a high osteogenic potential, whereas the fibrous layer showed negligible ALP activity. In addition, the cambium layer showed a higher cell density as compared to the fibrous layer, and the interface between the two layers was phenotypically distinctive.

Fig. 7. Histology (x400) of freshly harvested periosteum stained with ALP histochemical assay with hematoxylin counterstaining. The cambium layer shows a high level of ALP activities (represented by red color), whereas the fibrous layer shows negligible ALP activities.

As shown in Figure 8-A, immediately after the cell harvest (Day 0), the cambium layer cells showed statistically higher ALP activity than the fibrous layer cells. On Day 3, however, the ALP activity of the cambium cells dramatically decreased, but was still significantly higher than that of the fibrous layer cells. The ALP activity of the cambium layer cells slightly increased on Day 9, whereas that of the fibrous layer cells remained low, and no statistical differences were found among fibrous layer cells on Day 3, 6, and 9.

Although the cambium layer cells produced more osteocalcin than the fibrous layer cells on Day 3, no statistical difference was found between the cell types during the culture. However, the production of osteocalcin by both types of cells significantly declined from Day 3 to Day 9 (Fig. 8-B).

Periosteal Augmentation for Bone-tunnel Healing

Biomechanical Data

In all biomechanical pull-out tests, the failure took place at the interface between the tendon and the bone tunnel. The ultimate failure load and the tensile stiffness were used as measures of the structural properties of the tendon-bone interface. The average and standard deviation of the ultimate failure load of each group is summarized in Figure 9. According to a one-factor ANOVA, the effect of the treatment was significant. The subsequent post-hoc analysis revealed that Group B had higher ultimate failure load than any other groups and that it was statistically significant. There was no statistical difference in the ultimate failure load between Group A and Group D. Group C had the lowest ultimate failure load. The tensile stiffness was calculated from the linear region of the load-displacement curve. Although Group B showed the higher tensile stiffness than Group A and Group D, there was no statistically significant difference between these groups. The average tensile stiffness of Group C was significantly lower than any other groups.

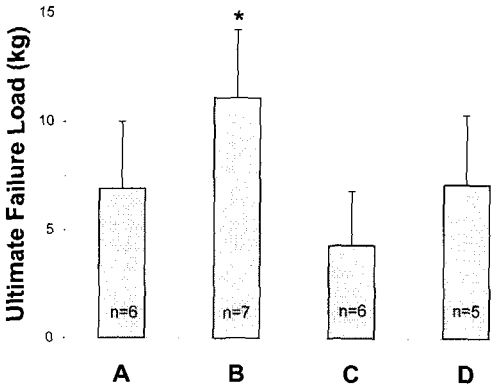
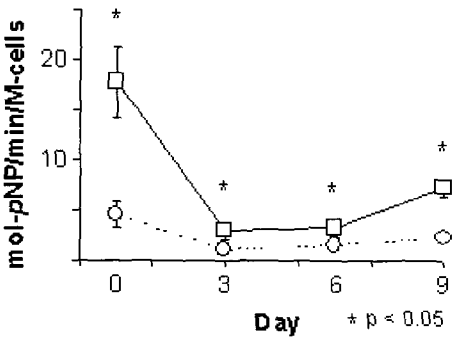
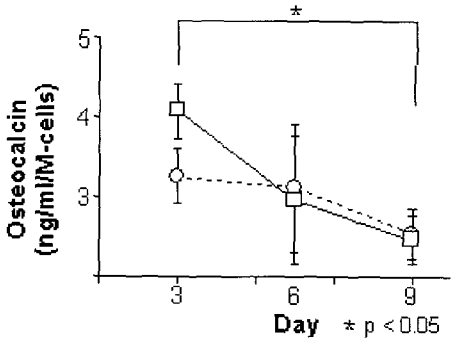


Fig. 9. Graph shows the ultimate failure load of the tendon-bone interface at 6 weeks after surgery. A = 6.9 ± 3.1 kg (n = 6), B = 11.1 ± 3.1 kg (n = 7), C = 4.4 ± 2.5 kg (n = 6), and D = 7.1 ± 3.2 kg (n = 5). * $p < 0.05$



(A)



(B)

Fig. 8. Quantitative ALP (A) and osteocalcin production (B) activities from the fibrous layer (○) and cambium layer (□) cells.

Histological Observation

New bone formation around the tendon graft in the bone tunnel was evaluated qualitatively using two fluorescent colors (Fig. 10). The green color (calcein at the first and second weeks after surgery) represents the bone formation during the early stage of the healing process, and the red color (alizarin complexone at the fifth and sixth weeks after surgery) indicates that of the latter stage during the healing process. Fluorescent histologic evaluation revealed that Group B had the most organized and significant bone formation around the bone tunnel. In particular, the new bone formation was established very tightly around the grafted tendon in Group B, whereas a large amount of granular tissue was present in the interfacial zone in the other groups (A, C, and D). It also was observed that the diameter of the bone tunnel and the tendon graft had reduced during 6-week period, indicating an active remodeling process of the tendon and bone during the healing period.

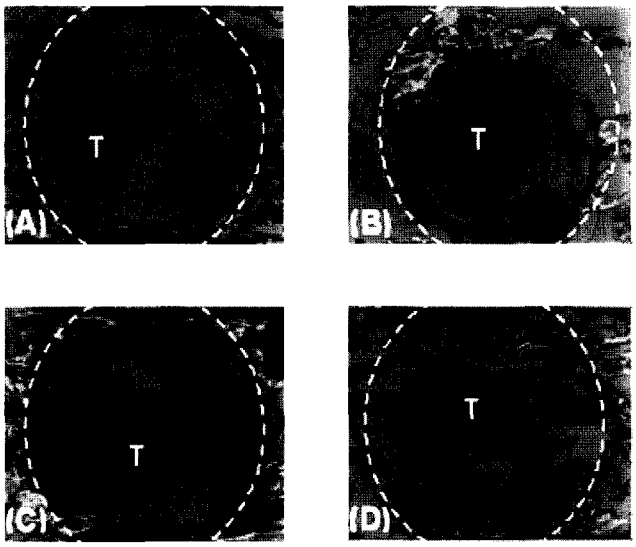


Fig. 10. Representative fluorescent histologies show calcein green (at 2 and 3 weeks) and alizarin red (at 5 and 6 weeks) staining from (A) Groups A, (B) Group B, (C) Group C, and (D) Group D. The white circle represents an approximated area of the bone tunnel created at the time of surgery. (T-tendon)

Standard histologic evaluation with hematoxylin and eosin provide more detailed descriptions of the general healing process at the tendon-bone interface. At 3 weeks after the surgery, Groups A and B showed a presence of the interposed periosteum consisting of the fibrous and cambium layers within the bone tunnel. In Group A and Group B, a newly formed woven bone with unorganized fibrous structure was observed at the juxtaosseous region around the periosteum under regular light and polarized-light microscopes. The bone formation in Group B (Fig. 11) seemed to be driven by the osteochondral ossification of the periosteal cambium layer, which was evident by the abundant presence of chondrocytes at the cambium layer-bone interface. In Group A, however, chondrocytes were not found near the juxtaosseous region, nor in the cambium layer. In Group D, the tendon-bone interface was mostly an interfacial tissue without a woven bone formation.

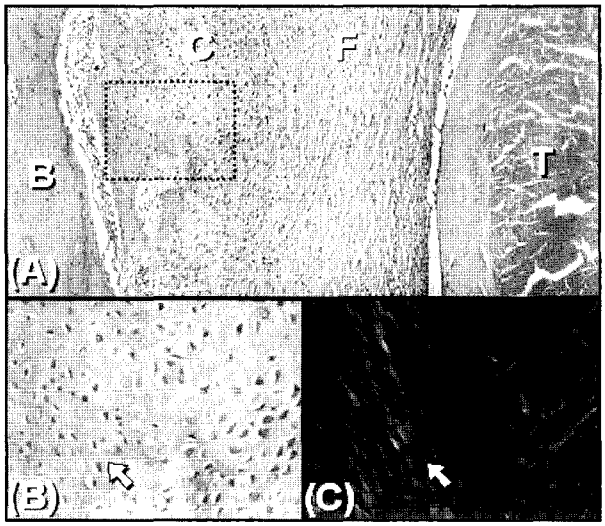


Fig. 11. Histological evaluation of a specimen from Group B at 3 weeks after surgery is shown (H&E, x200). B-bone; F-fibrous layer; C-cambium layer; T-tendon (B) Magnified image (x400) is shown from the dotted rectangular area in (A). Chondrocytes developed in the cambium layer. (C) Polarized light microscopic evaluation of the same area reveals a newly formed woven bone with unorganized structure (arrow).

At 6 weeks after the surgery, Group A and Group B showed a significant new bone formation with well-organized lamellar structure. In Group A, however, the tendon graft seemed to have had a rapid remodeling process. However, Group B showed a tight interdigitation between the tendon graft and the newly formed bone with abundant Sharpey's fibers (arrows in Fig. 12). In Group D, a new bone formation around the tendon graft also was shown, but a thick interfacial tissue with unorganized structure was evident around the tendon.

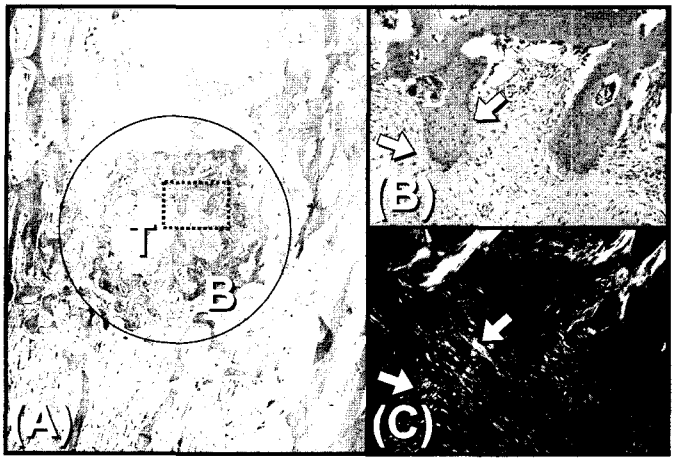


Fig. 12. (A) Histological evaluation of a specimen from Group B at 6 weeks after surgery is shown (H&E, x40). B-bone; T-tendon The circle represents the approximated location of the initial bone tunnel of 2.38-mm in diameter. (B) Magnified image (x200) is shown from the dotted rectangular area in (A). (C) Polarized light microscopic evaluation of the same area shows collagen fibers (arrows) interconnecting the newly formed bone and the tendon, which resemble the Sharpey's fibers.

DISCUSSION

In the current study, we were able to successfully harvest the fibrous and cambium layer cells separately using a sequential enzymatic digestion method without damaging or losing cambium layer cells, and cells from each layer showed different phenotypes in a standard monolayer culture. However, collagenase digestion time for each layer would be dependent on the thickness of each layer, as it varies with the age of specimen and the donor site [33, 34]. When seeded on a monolayer culture condition, the proliferation rate of the fibrous layer cells was much higher than that of the cambium layer cells, as confirmed by the BrdU immunostaining. In some samples, a small number of fibrous cells were found among the harvested cambium layer cells, and those fibrous layer cells quickly multiplied to form colonies and outnumbered the cambium layer cells in a short period of time. This is contradictory to a previous finding by Tonna and Cronkite [21] that the cambium layer had a higher cell proliferation than the fibrous layer *in vivo*. These contradictory findings between *in vivo* and *in vitro* results suggest that a monolayer culture of the mixed cell population from the entire periosteum may misrepresent the original differential cell populations of the two layered periosteum.

Interestingly, osteoblast-like cells harvested from the cambium showed a diverse BrdU staining and varying cell sizes in the monolayer culture system. A strong correlation was found between the cell morphology and the cell proliferation among cambium layer cells, in which small round cells showed the higher proliferation activity than large round cells. These can be explained by the general cell biology concept that a cell loses its proliferative potential during the differentiation and enlargement stage [35]. Ito et al. [36] also observed a similar non-homogeneous cell proliferation among the cambium layer cells in their periosteum explants cultured in agarose *in vitro*.

Initially the cambium cells demonstrated higher osteogenic characteristics (higher ALP and osteocalcin levels), as compared to the fibrous cells. However, these differences significantly diminished in two dimensional culture condition within a short period of time. This finding might indicate that the monolayer culture system may not be suitable to maintain the high osteogenic potential of the cambium layer cells in the periosteum. Nakahara et al. [14] reported that the harvested periosteum-cells showed a fibroblast-like morphology in monolayer cultures, and differentiated into osteoblasts or chondrocytes when inoculated *in vivo*. These findings suggest that the fibrous layer cells may have the potential to differentiate into osteoblast-like cells in certain environments.

In periosteum augmentation *in vivo* study, we observed similar founding with *in vitro* study, which the cambium layer has stronger osteogenic potential than the fibrous layer in periosteum and these two layers show contradistinctive manners phenotypically and functionally. The periosteal cambium layer had to be placed facing toward the bone to obtain the significant improvements in the mechanical strength and histological appearance at the bone-tendon interface. When the periosteal fibrous layer was placed facing toward the bone tunnel, the interface strength was only as good as that of the negative control group (Group D). Biologic functions of the periosteal fibrous layer still are uncertain in the current study, but the mechanical and histologic findings support a hypothesis that the fibrous layer in periosteum may have similar biologic characteristics as the tendon in a bone tunnel. Use of an inert periosteal patch to augment the tendon graft (Group C) resulted in the lowest interface strength among groups, suggesting that the inert periosteum failed to provide osteogenic stimulation to the tendon-bone interface. The fact that the interface strength of the inert periosteum group was lower than that of the negative control group (Group D) suggests that the inert periosteum might have blocked nutrient transport to the tendon graft and impeded biologic interactions between the tendon and the bone.

Several studies have suggested that biologic augmentation of the tendon-bone interface using a rhBMP would enhance the histologic and mechanical properties of the interface [15, 37]. However, the exogenous agent best suited for tendon-bone healing is currently uncertain. A similar paradigm incorporates the fact that periosteum is one of the natural, osteogenic organs existing in the body. Therefore, it is a reasonable approach to use periosteum as an

interface scaffold between the tendon graft and the host bone to enhance the tendon-bone attachment.

Liberated periosteal cells or freshly harvested periosteum is a popular tissue engineering material known to have chondrogenic and osteogenic potential for the repair of damaged musculoskeletal tissues. However, heterogeneous phenotypic characteristics and functional multi-potentiality of periosteum still remain unclear for clinical applications. The current study showed that cambium layer has stronger osteogenic potential than the fibrous layer in periosteum and these two layers show contradistinctive manners phenotypically (monolayer culture of periosteal cells) and functionally (periosteal augmentation for bone-tunnel healing). These findings suggest that extra care needs to be taken in order to identify and maintain the intrinsic phenotypes of the heterogeneous cell types within the periosteum. This will improve our understanding of periosteum in applications for musculoskeletal tissue repairs and tissue engineering. Further studies are necessary to investigate the specific role of each cell population in the milieu of osteogenesis or chondrogenesis *in vivo*, and long-term phenotypic characteristics of periosteal cells in various culture environments.

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