

Original

Evaluation of Osteogenic Potential of Cultured Periosteum Derived Cells -Preliminary Animal Study-

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Abstract: Periosteum is a source of osteoprogenitor cells and some investigators advocated the effective use of periosteum as a grafting material for the repair of bone and joint defects. In the present study, we showed bone formation induced from cultured periosteum-derived cells (CPDC) in the rat calvarial defect model. Periosteum taken from a rat tibia was immediately placed in culture medium with 10% fetal bovine serum. After confluence, periosteal cells were re-cultured three dimensionally on a collagen sponge for 7 days. Periosteal cell/collagen complex was grafted in calvarial defect of rats. At 30 and 60 days post grafting, grafted tissue was extracted and compared histologically and radiographically with control groups. In the experimental group at 30 days after implantation, new bone formation was seen. Radiographically, mineralized new bone formation was revealed in the defect at 60 days post grafting. New bone formation in periosteal cell/ collagen complex was greater than the other groups. Our results suggest that CPDC may have an important role in bone formation in the calvarial defect in rats.

Key words: Cultured periosteum-derived cells, Calvarial bone defect, Bone repair.

Introduction

Periosteum has a rich potential of osteo/chondrogenesis¹⁻⁴. Moreover, some investigators reported osteogenic potential of periosteal cell. Nakahara et al.⁵ described heterotopic ossification by cultured periosteal-derived cells (CPDC) harvested from chick tibia and grafted in the back muscle of nude mouse. Hanada et al.⁶ reported that cultured periosteum-derived cells from rat showed strong expression of osteogenic markers as BMP-2. We also reported new bone formation by periosteum from the tibia of rat autogenously graft into submandibular muscle.⁷ These results suggested the efficacy of the clinical application of CPDC for bone repair and joint defects. However, the osteogenic potential of cultured periosteal cells in bone defect is poorly understood. In the present study, we examined radiographically and histologically the osteogenic potential of CPDC grafted in the rat calvarial defect.

Materials and Methods

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Tissue preparation and cell isolation

Twenty-four 7-week-old (male or female) Sprague-Dawley rats purchased from Charles River (Osaka, Japan) were housed at 25°C and fed with standard animal diet (Oriental Co., Osaka, Japan) with water provided *ad libitum* for 1 week prior to grafting. Primary periosteal cells were isolated from a strip of periosteum taken from tibia (3 X 5 mm) under anesthesia with sodium pentobarbital (10 mg/kg b.w.) (Fig. 1A). The tissue was then placed in the cold phosphate buffered saline (PBS, Sigma Chemical Co., St. Louis, MO) containing 100 mg/ml penicillin G, 50 mg/ml gentamycin sulfate and 0.3 mg/ml fungizone. Finally they were washed three or four times with PBS.

Transplantation procedure

Animals were divided in three groups:
Group A: CPDC/collagen complex graft (n=10)
Group B: collagen without CPDC graft (n=10)
Group C: no graft (n=4).

Specimens in each groups were extracted at 30 or 60 days post grafting. Animals were cared in accordance with the Guidelines for Animal Research of Okayama University Dental School and with the principles of the Declaration of Helsinki. The study

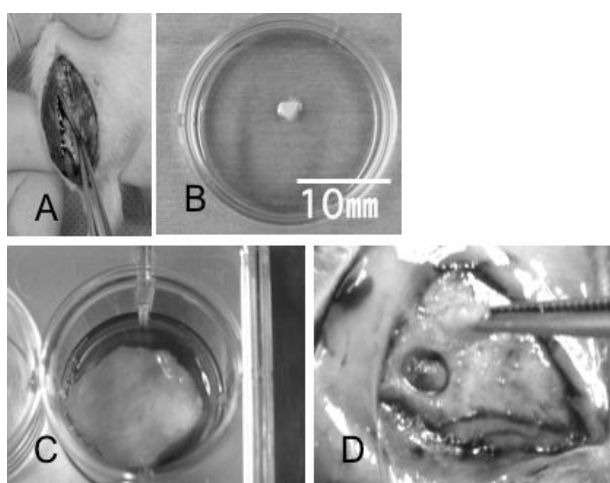


Fig. 1. Picture of surgical procedures. A: The removed periosteum from tibia of rat. B: The cultured of periosteum. The osteogenic layer facing to the dish surface. C: The collagen scaffold combined with cultured periosteum-derived cell (CPDC). D: The grafting material placed in the rat calvarial defect.

protocol was approved by the Ethics Committee of Okayama University.

Grafting procedure

Surgery was performed under anesthesia with sodium pentobarbital (10 mg/kg b.w.). After that, Calvarial defects were created. A periosteal flap was removed to expose the calvarial bone. A circular bone defect of 4-mm diameter was created using a round dental burr with normal saline solution. The defect was filled with grafting materials or without grafting (Fig. 1D), and then sutured with black silk. Penicillin G (400,000 U) was administered intramuscularly for three days postoperatively.

Histological observation

Rat calvaria was extirpated and fixed in 10% neutral buffered formalin before decalcification in 5% trichloroacetic acid for 7 days. Tissue samples were then dehydrated using a graded ethanol series and embedded in paraffin. They were cut at 6 mm, and stained with hematoxylin and eosin to be examined under light microscopy.

Radiographic evaluation of calcification in the defect

The specimens were also examined by radiography (Softex, 35mA, 5eV, 20 sec).

The evaluation of bone area in the defect

The area of new bone at 60 days after grafting in the calvarial defect was determined using Image Soft (Mac Scope; Mitani Shoji, Fukui, Japan) based on histological sections from the sagittally cut center of the defect stained with hematoxylin and eosin. Statistical analysis was evaluated using Mann-Whitney's U-test.

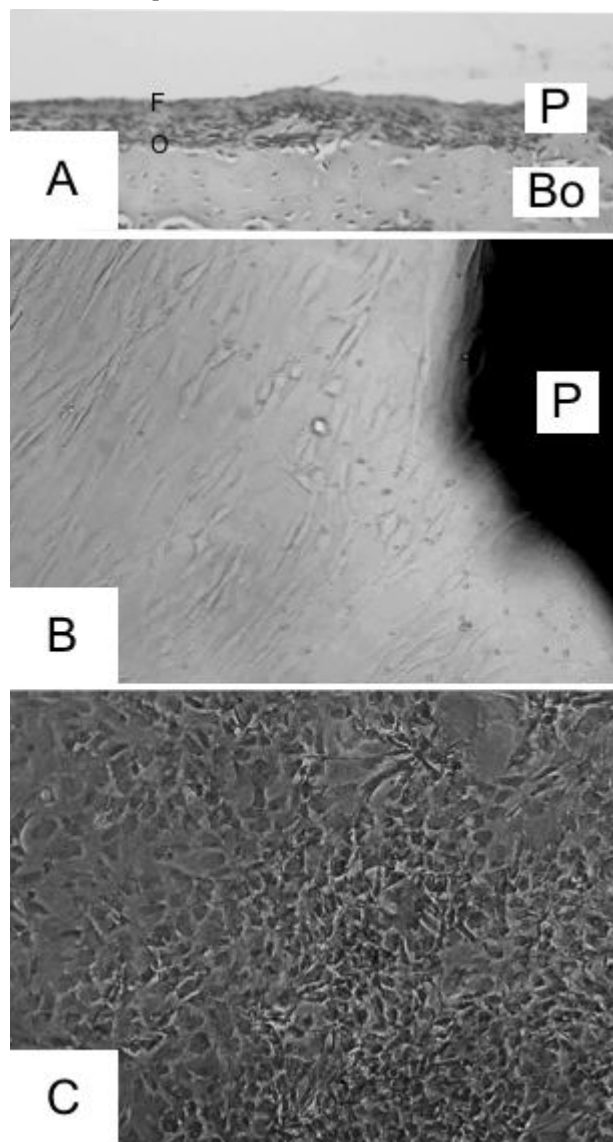


Fig.2. Pictures of the normal intact periosteum and cultured periosteum-derived cells (=CPDC). A: Histological examination of periosteum. Periosteum (P) covering bone (Bo) consists of osteogenic (O) and fibrous layers (F). B: Some periosteum-derived cells appear. P: cultured periosteum. C: The CPDCs are resuspended in osteogenic medium to shows osteogenic differentiation.

Results

Histological findings of periosteum

Intact normal periosteum covers the bone surface and consisted of two basic layers, fibrous and osteogenic layers (Fig.2A).

In vitro cultured periosteal cells

At 7 days after culture, periosteal cells migrated from the periosteum explant (Fig. 2B). These cells are fibroblast-like in appearance. At 14 days after culture, the periosteum-derived cells became confluent. After treated with osteogenic medium, the cells changed to cuboid-shaped cell. (Fig. 2C).

Calvarial defect post-grafting

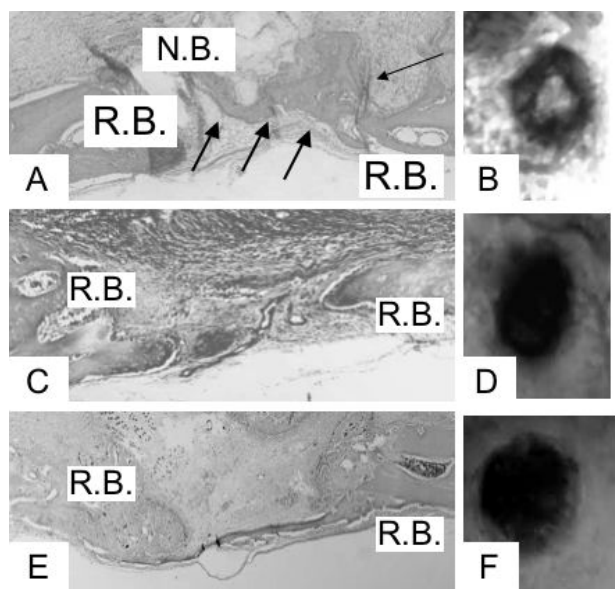


Fig. 3. Histological and radiographical findings of the calvarial defect at 60 days after grafting.

A: CPDC/collagen complex graft. Newly formed bone (N.B.) (arrows) is seen in the center of defect. R.B.: Residual bone. Hematoxylin and eosin. Mag. X 25. B: Softex finding of CPDC/collagen complex. New bone is seen in the center of the defect. C: Collagen graft. No new bone is seen in the center of the defect. Small bone (arrows) appeared at the edge of the residual bone. Hematoxylin and eosin. Mag. X 25. D: Softex finding of the defect. No new bone is seen in the center of defect. E: No graft group. No new bone is seen in the center of the defect. Hematoxylin and eosin. Mag. X 25. F: Softex finding of no graft group. No new bone is seen in the defect.

In the group A: At 30 days after grafting, new bone formation was detected at the center of the defect. Radiographically, small areas of calcification were seen in the defect. At 60 days post grafting, large amount of new bone was clearly observed in the defect, which was not fused to the residual bone (Fig. 3A). Radiographically, the calvarial defect was partially filled with radiopaque areas (Fig. 3B).

In group B at 60 days post grafting, no new bone was observed at the center of defect and only small areas of bone formation were seen at the periphery of the residual bone (Fig. 3C). Radiographically, presence of radiopaque areas was detected only at the periphery of the defect (Fig. 3D).

In group C at 60 days post grafting, evidence of fibroblastic cells without bone formation in the defect was observed (Fig. 3E). Radiographically, no calcification was seen in the defect (Fig. 3F).

Evaluation of calcified area in the defect

The new bone area in the defect at 60 days after grafting was significantly ($P<0.01$) largest in the group A (35 ± 5.11), compared with groups B (3 ± 2.01) and C (5.1 ± 2.66) (Fig. 4).

Discussion

In the present study, we suggested the osteogenic potential of

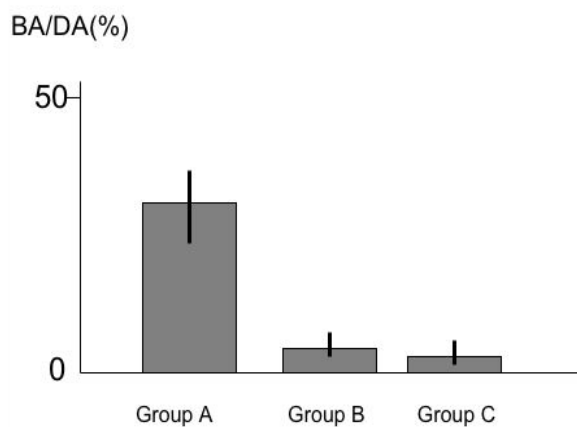


Fig. 4. The Bone area (%) in the calvarial bone defect in each group

CPDC in the rat calvarial defect model. Although some previous studies stated the osteogenic potential of the cultured periosteal cells in the blood supply rich circumstances such as muscles^{5,6}, very few studies have reported the osteogenic potential of cultured osteogenic cells derived from periosteum in the bone defect.⁸

Osteogenic potential of periosteum is widely proven by the research of the histological process of bone fracture healing or bone formation from periosteum graft.^{2,3,5} These researches revealed that osteoprogenitor cells of the osteogenic layer play a very important role of osteogenesis. Therefore, we placed the periosteum with osteogenic layer facing the culture-dish surface in accordance with Hanada et al.⁶ giving high possibility of osteoprogenitor cells from osteogenic layer of the periosteum.

Groger et al.⁸ reported the efficacy of CPDC with bioabsorbable polymer fleece for the repair of mandibular critical sized defect in the minipigs. Takushima et al.⁹ demonstrated the enhancement of osteogenic potential by transplantation with CPDC in the distracted bone gap in rabbit experimental model. In our previous study⁷, periosteal cells proliferated and differentiated into chondrocytes and formed new bone via endochondral ossification in the whole graft of periosteum. However, in the present study, cultured periosteal cell showed direct osteogenic differentiation. We used osteogenic medium, which contains ascorbic acid, β -glycerophosphate, and dexamethasone,^{6,7,9,10} This medium might prompt direct osteogenic differentiation of cultured periosteal cell. Groger et al.⁸ using same osteogenic factors, reported that cultured periosteal cells showed direct osteogenic differentiation in minipig animal model.

In the present study, we successfully used 3-D collagen sponge as scaffold for bone formation. There have been many different processing techniques to develop the design and fabricateion of scaffold in order to deliver stem cells for tissue regeneration^{11,12,13}. The scaffold for osteoprogenitor cells is required to promote progenitor cell migration, proliferation, and differentiation, as well as vascularization. Imamura et al.¹⁴ used a collagen scaffold as three-dimensional cell culture with pluripotent embryonic stem

cells to be capable to differentiate into hepatocytes for liver repair in the animal study. For that reason, the collagen sponge appeared to be a suitable material to deliver osteogenic cells into the defect.

This CPDC grafting technique may enable the minimum invasion therapy to reconstruct oral and maxillofacial osseous defect. Although, repair of bone defect by autogenous cultured osteoprogenitor cells could maintain their osteogenic potential after *in vivo* grafting, the new bone induced from CPDC did not fill the bone defect. The calvarial has mainly the compact bone with few bone marrow. Therefore, the blood supply from around tissue was not sufficient to prompt the osteogenesis of the grafted material. In addition, we reported the whole graft of the immediately removed periosteum showed 65% bone formation in the same rat calvarial defect¹⁵⁾. These results may suggest that periosteum graft involved osteogenic cell and vascular endothelial cells worked as a highly potential graft to form new bone in the bone defect compared with CPDC graft.

Furthermore, more studies to enhance bone formation including combination with bone growth factors like the report about BMPs application described Kishimoto et al.¹⁶⁾ will be expected to prompt this periosteal cell grafting for clinical use.

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