Non-vascularized bone isograft using GFP transgenic mice

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Purpose: To further understand the viability of the non-vascularized bone graft and the origin of the bone-forming cells, we have developed a novel mouse femur model that permits direct-viewing analysis of bone graft healing.
Materials and Methods: Critical size defects were prepared in one side of femur of 15 C57BL/6 mice and repaired by non-vascularized femur with intact periosteum and parts of muscles from isogenous GFP transgenic C57BL/6 mice. Sacrifice was performed 3 days, 1, 2, 3, and 4 weeks after surgery for histological and fluorescence microscopy analysis.
Results: Healing process of the transplants is similar to that of bone fracture. The osteoblasts and newly formed trabeculae which were beneath the grafted periosteum showed green fluorescent protein (GFP) expression, which was strongly expressed in the transplanted muscles.
Conclusion: These data demonstrate that part of non-vascularized grafted bone can keep viability and has the potential of osteogenesis, indicating an important role for the grafted periosteum and bone in new bone formation. **(Int Chin J Dent 2007; 7: 87-91.)**
Key Words: GFP transgenic mice, isograft, non-vascularized bone graft.

Introduction

Non-vascularized bone graft, as an effective method for the repair of bone defects, is widely used in the world. Conventional view of incorporation of non-vascularized bone graft to the recipient is called creeping substitution.[1] The grafts will finally be totally replaced by the host and all bone-forming cells originate from the recipients.[2] However, some researchers hold a contrary opinion. Ray and Sabet[3] used a radioactive tracer as a bone label to perform bone transplantation between highly inbred mice. And Arora et al.[4] identified sex chromatin following sex-mismatched bone transplantation between highly inbred rabbits. They found that not all the grafted bone suffered from creeping substitution, but a few parts of them could keep its viability. Kakinoki et al.[5] transferred non-vascularized tibia grafts from male to female rats, then extracted genomic DNA and made Sry-specific PCR detection. Their results demonstrated that a small number of grafted bone survived and proliferated over time. However, these methods have low sensitivity, much complication; they have provided limited understanding of transplanted cells.

Green fluorescent protein (GFP) is a 27-KD protein, originated in jellyfish. It provides a technically assessment of transgene activity and can be viewed as a real-time image in living tissues. Recently, Jiang et al.[6] reported that GFP activity can be preserved in paraffin sections for standard fluorescence microscopy. Thus, it is hypothesized that GFP transgenic mouse as transplantation donors would be an easier way to determine the origin of newly forming cells and comprehend clearly the behavior of grafted cells during the repair process of bone defects.

In the present study, we transplanted non-vascularized femur grafts from GFP transgenic mice to its isogenous mice. Fluorescence microscopic and histological assay were performed at 3 day, 1, 2, 3, and 4 weeks after surgery, to identify the viability of the grafted bone and the origin of the bone-forming cells. This study may be of importance in the future development of clinical methods to reconstruct large mandible defects using

non-vascularized bone grafts with dental implants at the same time.

Materials and Methods

The study procedure was approved by the animal center of Shan'xi province. All animal experiments were performed according to the guidelines of the animal committee of the Fourth Military Medical University.

Surgery procedure

Fifteen young adult male C57BL/6 mice (weighing 30-50 g) and fifteen 12-week male GFP transgenic C57BL/6 mice (weighing 25-30 g) were used in this study. All surgeries were performed under general anesthesia with 1% sodium pentobarbital by intraperitoneal injection. A standardized metaphyseal bone defect of the femur (5 mm long, full thickness) was created in C57BL/6 mouse. Then a 5-mm-long-femur with intact periosteum and parts of muscles was harvested from GFP transgenic C57BL/6 mouse. The graft was carefully placed into the defect of the C57BL/6 mouse and fixed with sutures. Wound was closed in layers and fixed with bandage (Ex vivo time of the graft was less than 15 minutes). Animals were sacrificed at 3 days, 1, 2, 3, and 4weeks after surgery respectively and the grafts were carefully harvested and fixed immediately into 10% phosphate-buffered neutral formaldehyde for 24 hours.

Histology

For histology, the specimens were demineralized in 0.1 mol/L EDTA for 2 weeks, dehydrated in graded ethanol solutions, embedded in paraffin. Serial sections were cut in 4-μm-thick at different levels sufficiently far apart (about 200 μm) to avoid replicate sampling of a single surface event. Sections were then mounted on glass slides and stored at -20˚C away from light. Finally, some sections were stained with hematoxylin-eosin (H-E) and Masson trichrome, whereas others were left unstained for evaluation under fluorescence microscopy.

Fluorescence microscopy

All sections were observed under fluorescence microscopy. The average exposure time was 1-1.5 s, and the excitation light was 488-530 nm blue light.

Results

All animals remained healthy during the healing period and healed uneventfully. Infection and immunological rejection were not observed.

Histology

Figs. 1-7 show histological findings (NB, new bone; GB, grafted bone; M, muscles; and OB, original bone).

Three days: There were lots of blood clots and inflammatory cells in the grafted areas. Transplanted bone was partly absorbed and a thin layer of newly formed immature trabecular was on the surface of them.

One week: Blood clots had been replaced by vascularized granulation tissues which extended all the way to the grafted bed. The union between the grafted bone and recipient bone was full of mesenchymal cells undergoing chondrocytic differentiation. Extensive bone absorption was observed at the periphery of the bone graft indicated by the numbers of osteoclasts.

Two weeks: The grafted areas could be divided into five layers according to different tissues: grafted muscles, grafted periosteum, newly formed cartilage, newly formed trabeculae, and grafted bone. Muscle fibers interlacing with each other were in the outside of the grafted areas. Their cellular nuclei were on the margins of the fibers. Connecting with muscle fibers, periosteum was intact and numbers of mesenchymal cells aggregated

in its germinal layer. Beneath the periosteum, there were lots of chondrocytes which seemed big and more karyokinesis. Newly formed woven trabeculae occupied nearly two thirds of grafted areas, and numerous osteoblasts lined around them. One third of the cortex of grafted bone had been absorbed and new bone formation occurred on the superficial of the remains. Grafted bone marrow was almost intact and within it lots of lymphocyte and leukocytes got together.

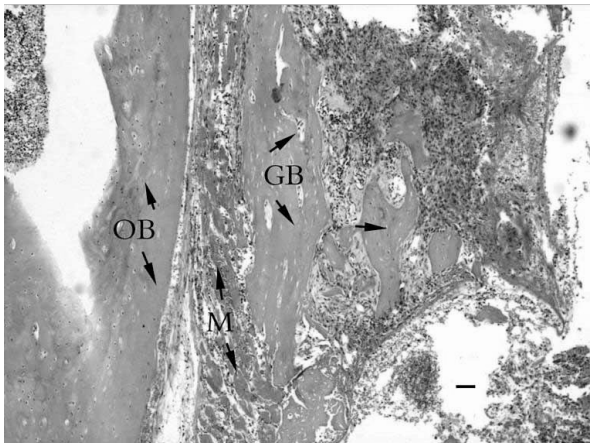

Fig. 1. Three days after surgery (H-E; Bar, 100 μm).

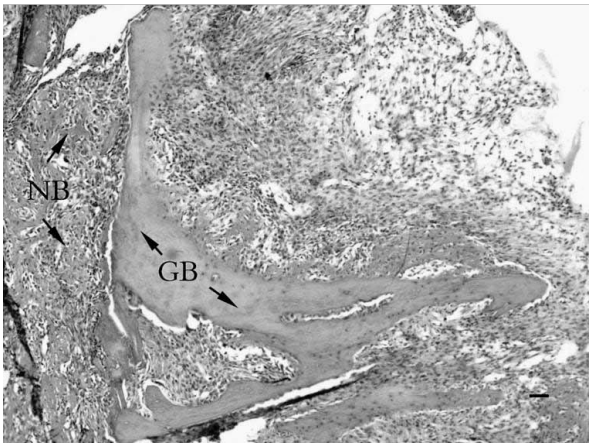

Fig. 2. One week after surgery (H-E, Bar, 100 μm).

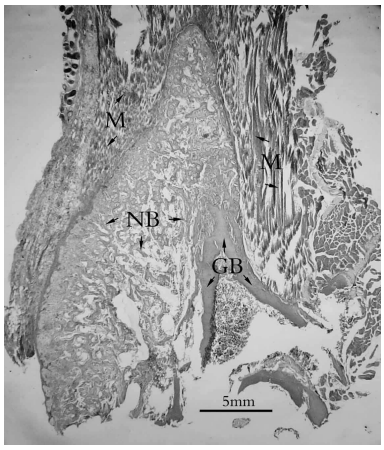

Fig. 3. Two weeks after surgery. **(**Masson trichormes)

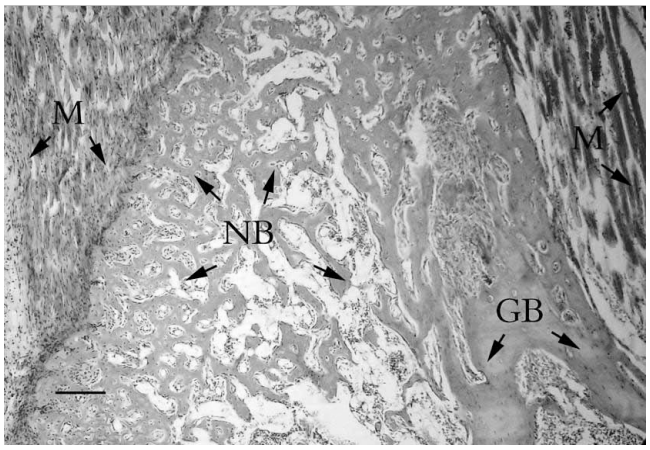

Fig. 4. Two weeks after surgery (H-E; Bar, 100 μm).

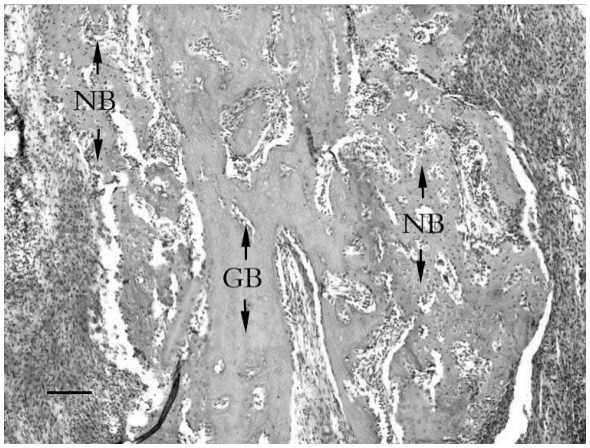

Fig. 5. Three weeks after surgery (H-E; Bar, 100 μm).

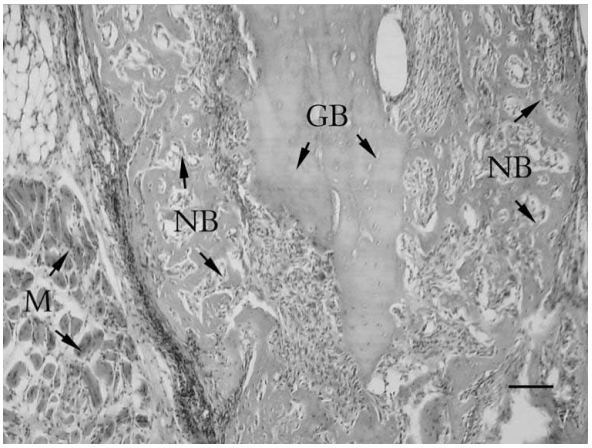

Fig. 6. Four weeks after surgery (H-E, Bar, 100 μm).

Three weeks: The remained grafted bone began to integrate with newly formed mineralized trabeculae. New

medullary cavity occurred in the area initially occupied by the bone grafts.

Four weeks: Newly formed bone incorporated to the grafted bone and it's difficult to distinguish them.

Fluorescence microscopy

Bright green light was found in transplanted muscles all the time under fluorescent microscopy (Fig. 7). The osteoblasts and newly formed trabeculae, which were beneath the grafted periosteum, also showed GFP expression.

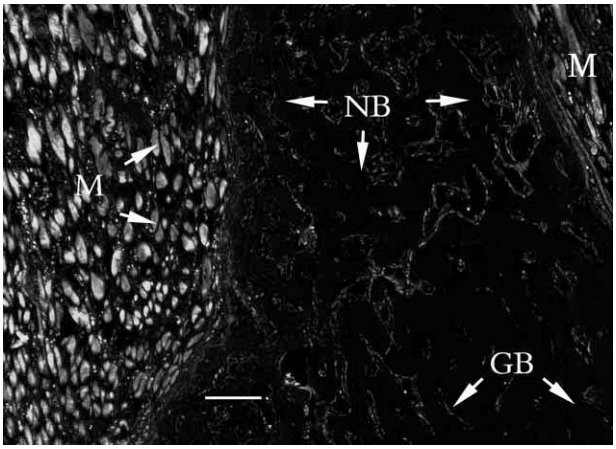

Fig. 7. Two weeks after surgery (Bar, 100 μm).

Discussion

Although the theory of creeping substitution has been introduced for more than one hundred years, there are still two questions not very clear: Does all the grafted bone be replaced by the newly formed bone? And where are the osteoblasts or osteocytes in the new forming bone from?[7,8] By now, large number of researches support that parts of the grafted bone will survive and have the ability of osteogenesis.[9-12] In this study, we investigated the viability of the non-vascularized grafted bone and its ability of new bone formation using isografting from fluorescence specific GFP transgenic mice to its isogenous normal mice. Our results suggest that the transplants can keep their viability and have the ability of osteogenesis. Although large part of the grafted bone was absorbed, the remains still kept its structure. Transplanted muscles and periosteum showed their viability by positive expression of green lights. Green lights in the newly formed trabeculae and osteoblasts revealed that bone forming cells were from the transplants. Thus our results indicate that the grafted bone and periosteum play an important role in the process of new bone formation, and that the transplant can keep its viability during the reparative process.

Owing to the early revascularization, transplanted bone, periosteum and muscles could keep their vitality and medullary cavity of grafted bone could keep its structure, as the femur cortex of mouse is thin, which facilitates blood vessels going through. Early revascularization was established by the blood-abundant recipient bed and the transplanted soft tissues which can get blood easier than bone. Femur cortex of mouse is about 1 mm thick, through which blood vessels go easily. No fixation in medullary cavity could kept its structure and conduce to revascularization. Healing process of transplants is similar to the healing process of bone fracture.

In this study, we used GFP transgenic C57BL/6 mice as donor animals. These animals are very special with green muscles and bones in the sunlight and green eyes and ears under violet light. Histologically, muscles show bright green light under fluorescence microscopy. To bone, some researchers once worried that the demineralization might destroy the expression of GFP. Recently, Jiang et al.[6] has detected positive green expression in demineralized bone sections. It is very interesting that positive expression only confine in osteoblasts and at the margin of the bone instead of the whole bone. Our study used 0.1 mol/L EDTA for 2 weeks' demineralization, and the results indicated that GFP can be preserved in paraffin sections for standard fluorescence microscopy. It is very helpful to use GFP transgenic mouse to be an animal model to study non-vascularized bone graft.

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