DIRECT BONE INDUCTION IN THE SUBPERIOSTEAL SPACE OF RAT CALVARIA WITH DEMINERALIZED BONE ALLOGRAFTS

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KEY WORDS
Bone induction
Demineralized bone
Bone morphogenetic protein

In order to clarify the understanding of bone induction with crude bone morphogenetic protein (BMP)-containing allografts in subperiosteal conditions, chondrogenesis and osteogenesis were histologically evaluated following the implantation of demineralized bone (DB) in the subperiosteal space of calvaria of 30 Wistar rats. On the forehead of the rat, DB particles were placed onto the denuded calvarial bone and covered by skin-periosteum flaps without any perforations of the marrow spaces of the calvaria. Sintered hydroxyapatite particles (HA) were also placed as a control. In the DB group, new bone formation on the surface of calvaria was achieved between 2 and 8 weeks after the operation. However, no chondrogenesis was seen throughout the experimental period. In the HA implantation group, fibrous tissue encapsulation of HA particles was generally seen. These results suggest that DB containing crude BMP might have the capacity for direct osteoblast induction from undifferentiated mesenchymal progenitor cells in vivo in specific situations, that is, in a subperiosteal space of uninjured rat calvaria.

INTRODUCTION

Osteointegrated dental implants have been widely accepted as a predictable treatment for totally or partially edentulous patients for rehabilitation of mastication and esthetics. However, because of a lack of adequate bone, dehiscences and fenestrations frequently occur around dental implants. Therefore, several studies of the treatment of peri-implant defects have been performed using autologous demineralized bone (DB). DB has been proven to induce cartilage and bone formation in various tissues in vivo. The results suggest that the biological sequence of DB-induced bone formation strongly resembles that of normal endochondral ossification. It is generally accepted that the osteogenic potential of DB is derived from bone matrix proteins, especially from members of the transforming growth factor β (TGF-β) superfamily, that is, bone morphogenetic protein (BMP) and TGF-β might be the dominant factors of osteoinduction. Noda and Camilliere have showed in vivo stim-
ulation of bone formation by subperiosteal TGF-β injection onto the uninjured rat calvaria. These authors have found no evidence of cartilage interactions coinciding with bone formation of the type that occur in intramembranous ossification. In contrast, Joyce et al reported in vivo stimulation of chondrogenesis and osteogenesis by TGF-β injection of the type that occurs in endochondral ossification. Except for their decision to inject the rat femur rather than the rat calvaria, the experimental design of Joyce et al was quite similar to the study on bone formation by Noda and Camilliere. These studies suggest that TGF-β injected subperiosteally onto the femur may stimulate chondrogenesis followed by osteogenesis, and that the TGF-β injected subperiosteally onto uninjured calvaria may only stimulate osteogenesis. In effect, the same growth factors may play a different role in vivo if implanted in different sites or under different conditions. The present study was, therefore, designed to determine whether or not subperiosteally implanted DB plays a role in stimulating new bone formation without chondrogenesis.

**MATERIALS AND METHODS**

DB particles were prepared from the long bones of male Wistar rats by the method previously described. The bones were harvested, soft tissues were removed, and the bone marrow was discarded. The bones were cut into small pieces, demineralized in 0.5 M HCl (25 mEq/g) for 3 hours, and then washed with copious amounts of water for 2 hours, with absolute ethanol for 1 hour, and with ethyl ether for 0.5 hour. DB chips were lyophilized and crushed with hammer blows. The crushed bone chips were sieved to attain a particle size of 100–250 μm. HA particles (Bonelite Periopack; particle size, 300–500 μm; Mitsubishi Material Inc, Tokyo, Japan) were used as the bone nonosteinducible control. These particles were mixed individually with gelatin to make an approximately 3 × 6 × 2 mm rectangular pellet form for subperiosteal implantation. The study protocol was approved by the Hiroshima University School of Dentistry Committee on Animal Care. Thirty 8-week-old male Wistar rats were used in this study. Before surgery, the animals were sedated with pentobarbital and atropine. The forehead of each animal was shaved and sterilized with iodine solution. A 1.5-cm frontal incision was made, traversing the midline, and the skin-periosteum flap was gently reflected occipitally with a periosteal elevator (fabricated from a Bard-Parker 12 scalpel) to create adequate space for implantation. A DB pellet was placed in 15 animals, and an HA pellet was placed in the other 15 animals. The pellet was placed in the midline of the calvarial roof between the temporal protruberances. The skin-periosteum flap, which completely covered the pellet, was repositioned and sutured with interrupted 4-0 silk sutures for primary closure. The animals were allowed to drink tap water and were fed a standard diet (laboratory pellets) ad libitum. At 2 weeks, 4 weeks, and 8 weeks after surgery, animals were sacrificed, and a tissue block containing the pellet and calvaria with the surrounding soft tissue was obtained. Each specimen was fixed in 10% neutral buffered formalin, decalcified in K-CX solution (Fujisawa, Tokyo, Japan), and then dehydrated and embedded in paraffin. Serial sagittal sections were stained with hematoxylin and eosin and examined by light microscopy.

**RESULTS**

In general, wound healing of all animals was uneventful. Macroscopically, tubera were created by the pellet in the forehead of the animals immediately after surgery. However, 1 week after surgery, 80% of the volume of the tubera in all animals had diminished, and tubera were not detected at all after 8 weeks. No clear difference was seen clinically between the DB and the HA implantation groups.

Histological analysis did differ between the groups. In the DB group, 2 weeks after surgery, osteogenesis was seen to take place around the DB particles that were located close to calvarial bone in most animals. However, no chondrogenesis was detected in any animals (Fig 1). After 4 weeks, newly formed bone was seen around the DB particles, and no cartilage tissue was observed (Fig 2). Eight weeks after surgery, vascularized mature bone with bone marrow was formed adjacent to the original surfaces of the calvaria (Fig 3). The volume of bone neogenesis appeared to be less than the original volume of the implanted DB pellets because of the strong tension of the skin of the forehead, which compressed the DB pellets in the subperiosteal space during its degradation process. However, active, newly formed bone ingrowth into the DB particles was seen in most specimens. In the HA group, a dense network of granulation tissue was seen around the HA, connecting neighboring particles 2 weeks after surgery. Neither active bone formation nor osteoblastic activity was observed (Fig 4). Four weeks after surgery, minute amounts of newly formed bone were found close to the original surface of the calvaria in some specimens. However, overall findings in the HA specimen showed little or no bone formation (Fig 5). No ingrowth of bone around the HA particles was found 8 weeks postsurgery, and the HA particles were generally encapsulated by dense fibrous connective tissue (Fig 6). Both DB and HA pellets were partially degraded and appeared to be compressed in all groups. No inflammatory responses persisted at 8 weeks after surgery.

**DISCUSSION**

In this study, bone neogenesis and new bone formation were clearly presented in the DB implantation group with no chondrogenesis or cartilage formation. In the HA implantation group, which was the control for noninducible bone formation, little or no bone formation was detected, and the HA particles were generally surrounded by dense fi-
brous connective tissues. These results have strongly demonstrated that it is necessary for bone matrix proteins to be present for new bone formation to occur in nonperforated subperiosteal marrow spaces of calvaria. Moreover, it is noteworthy that neither chondrogenesis nor cartilage tissue were observed in any specimens throughout the healing period. The results may demonstrate that DB, which represents a crude BMP-containing allograft, can...
induce direct bone formation from undifferentiated mesenchymal cells in vivo.

Several investigators have shown the bone-inducing properties of DB allografts; DB has been proven to induce cartilage and bone formation in vivo. The biological sequence of DB-induced bone formation strongly resembles that of normal endochondral ossification. The osteogenic potential of DB is known to be derived from bone matrix proteins, mainly BMPs. It is generally accepted that BMPs (except for BMP-1) are members of the TGF-β superfamily that has 8 subtypes. DB contains all of the BMPs, as well as other cytokines in unknown proportions, and is referred to as 'crude' BMP-containing allograft. Recently, Katagiri et al. reported the potential of BMP-2-directed differentiation of undifferentiated mesenchymal cells into osteogenetic cells. Noda and Camilliere showed in vivo stimulation of bone formation by subperiosteal TGF-β injections onto uninjured rat calvaria. According to these investigators, no cartilage associated with bone formation was seen. In contrast, Joyce et al. used TGF-β injections to show in vivo stimulation of chondrogenesis and osteogenesis of the kind that occurs in endochondral ossification. Though their experimental design was similar to the design of the study by Noda and Camilliere, Joyce et al. used the rat femur for the injection sites, rather than rat calvaria. The cumulative observations of these studies suggest that the DB, implanted subperiosteally onto calvaria, may stimulate undifferentiated mesenchymal cells to differentiate directly into osteoblasts.

In our study, no chondrogenesis was observed, and osteogenesis was actively achieved with DB pellets. The non-osteoinducible control, HA, was surrounded by connective tissue. We propose two possible reasons why DB showed a different character when compared with former studies. The first reason may be related to the properties of undifferentiated mesenchymal cells in the calvarial periosteum. BMPs and other cytokines stimulate the undifferentiated cells to differentiate and proliferate. The undifferentiated cells can be divided into several types, and some of them were only differentiated to osteoblasts, not to chondrocytes, by BMP-2. Therefore, undifferentiated mesenchymal cells in the calvarial periosteum may differ from undifferentiated mesenchymal cells in other tissues. Although the undifferentiated mesenchymal cells in the calvarial bone developed intramembranous ossification, they may have had no potential to differentiate to chondrocytes. The second reason appears to be the in vivo activity of BMPs and other bone-derived growth factors that may interact with other cytokines released from various types of cells located in the mucoperiosteal tissue and derived from the bone marrow. Previous studies, which showed DB-induced endochondral ossification, were performed in different sites from this study, that is, by subcutaneous implantation or by subperiosteal implantation of injured bones. In this study, a concerted effort was made to avoid injury of the periosteum and bone surface. Therefore, successful stimulation of isolated DB beneath the muscle that created a subperiosteal space (a unique environment) produced a result different from those found in previous studies.

The results of the present study might demonstrate that the DB, which represents crude BMP-containing allograft, can induce bone directly from undifferentiated mesenchymal cells in vivo. From the limited results of this study, we may conclude that if DB is implanted in the subperiosteal space and isolated from the muscle and bone marrow, direct induction of osteoblasts could be achieved. A clinical problem such as pseudoarthrosis is caused by undesirable bone formation; this bone formation is sometimes retarded by cartilage, which can interfere with calcification and osseointegration. Therefore, this in vivo study illustrates the possibility of a controlled bone formation process whereby cytokines can be induced to osteogenesis without the intermediate stage of chondrogenesis. Further study should be encouraged to clarify the distinct mechanism of direct bone induction in the subperiosteal space using a membrane technique.

References

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