Original article

Osteoinductive potential of small intestinal submucosa/demineralized bone matrix as composite scaffolds for bone tissue engineering

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Background: Demineralized bone matrix (DBM) is extensively used in orthopedic, periodontal, and maxillofacial application and investigated as a material to induce new bone formation. Small intestinal submucosa (SIS) derived from the submucosa layer of porcine intestine has widely utilized as biomaterial with minimum immune response.

Objectives: Determine the osteoinductive potential of SIS, DBM, SIS/DBM composites in the *in vitro* cell culture and *in vivo* animal bioassays for bone tissue engineering.

Materials and methods: Human periosteal (HPO) cells were treated in the absence or presence SIS, DBM, and SIS/DBM. Cell proliferation was examined by direct cell counting. Osteoblast differentiation of the HPO cells was analyzed with alkaline phosphatase activity assay. The Wistar rat muscle implant model was used to evaluate the osteoinductive potential of SIS, DBM, and SIS/DBM composites.

Results: HPO cells could differentiate along osteogenic lineage when treated with either DBM or SIS/DBM. SIS/DBM had a tendency to promote more cellular proliferation and osteoblast differentiation than the other treatments. In Wistar rat bioassay, SIS showed no new bone formation and the implants were surrounded by fibrous tissues. DBM demonstrated new bone formation along the edge of old DBM particles. SIS/DBM composite exhibited high osteoinductivity, and the residual SIS/DBM was surrounded by osteoid-like matrix and newly formed bone.

Conclusion: DBM and SIS/DBM composites could retain their osteoinductive capability. SIS/DBM scaffolds may provide an alternative approach for bone tissue engineering.

Keywords: Demineralized bone matrix, human periosteal cells, small intestinal submucosa, osteoblast differentiation.

Tissue engineering has emerged as a promising approach for the repair and restoration of damaged bone. Bone tissue engineering requires a scaffold for cell attachment and maintenance of cell function, together with a rich source of osteoprogenitor cells in combination with osteoinductive growth factors [1].

The regeneration of new bone depends upon an appropriate three-dimensional scaffold capable of supporting bone formation. Naturally-derived biomaterials have been largely used to construct porous scaffolds with good biocompatibility and biodegradability [2-4].

Small intestinal sub-mucosa (SIS) originated from the sub-mucosal layer of porcine small intestine has been extensively utilized as biomaterial with minimum immune response [5-7]. SIS is composed of types I and III collagens beyond 90% and small amounts of types IV, V, and VI collagens [8]. SIS comprises a number of bioactive factors, such as basic fibroblast

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growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), transforming growth factor- β (TGF- β), and vascular endothelial growth factor (VEGF) as well as glycosaminoglycan, fibronectin, laminin, heparin sulfates, type IV collagen and hyaluronic acids [9, 10]. These constituents are well known to play an essential part for tissue engineering and remodeling. SIS has been used in practical biomedical fields, such as the repair of numerous body tissues, including musculotendinous structures, urinary bladder reconstruction, vascular reconstruction, and the structural support of tissue-engineered bone formation [11-13]. Therefore, SIS could be a biomaterial for tissue regeneration since it can stimulate cellular differentiation and advocate rapid host tissue ingrowths.

Demineralized bone matrix (DBM) represents a promising candidate for bone tissue engineering composite scaffolds due to its similar relation in structure and function with autologous bone [14]. The capability of DBM to promote osteoblast differentiation of mesenchymal stem cells has been recently shown and is believed to be attributed to the interaction of osteoprogenitor cells with these matrix-contained osteoinductive proteins, which can activate mesenchymal stem cells into osteoblasts [15]. Marshall Urist first identified and characterized in 1965 an osteoinductive substance while preparing a soluble extracts from demineralized bone [16]. Ultimately, this investigation resulted in the identification and production of bone morphogenetic proteins (BMPs). DBM, obtained from native osseous tissue, contains bone morphogenetic proteins and matrix proteins. BMPs are potent bone inductive growth factors, whereas matrix proteins, mostly collagens, provide an osteoconductive matrix [14, 17]. Despite the widespread clinical use of DBM, the mechanisms by which SIS/DBM composites induce osteoblast differentiation and lead to new bone formation remain poorly understood. Moreover, it has been postulated that SIS/DBM composites act as osteoinductive and/or osteoconductive materials. To date, the propensity of SIS/DBM composites to promote osteoblast differentiation have not been determined and characterized. Whether SIS/DBM composites could be served as novel scaffolds for bone tissue engineering is an issue that merits further investigation.

In this study, we investigated the osteoinductive potential of SIS/DBM to be used in bone tissue

engineering strategies by examining their capability to stimulate osteoblast differentiation of human periosteal cells *in vitro* and bone forming abilities *in vivo*.

Materials and methods

This study was conducted in accordance with the Declaration of Helsinki, and the study design and consent form were approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University.

Preparation of small intestinal submucosa

Porcine small intestinal sub-mucosa (SIS) was harvested from the small intestinal of healthy pigs within 4 h after killing. The procedure of processing SIS was in accordance with the protocol described by Badylak et al. [18] with some modifications. Briefly, fat was firstly removed from porcine jejunum, followed by a careful wash with water. The porcine jejunum was cut in lengths of 10 cm and then washed with a saline solution. SIS was obtained from mechanical removal of tunica serosa and tunica muscularis. Lastly, the obtained SIS was washed again with a saline solution and freeze-dried at -80°C for 48 hours using lyophilizer (Christ, Alpha. 1-4, Osterode, Germany). The dried SIS was then pulverized into fine powder using cutting mill.

Preparation of demineralized bone matrix

Freeze-dried human cortical and cancellous bones from donors (the age of 15-65 years) were ground by impact fragmentation and separated using sized sieves. Ground bone matrix (particle size less than 1,000 microns) was demineralized by exposure to diluted hydrochloric acid. Briefly, ground bone matrix was exposed to 0.5 N HCl (100 mg DBM to 10 mL 0.5 N HCl) and demineralized bone matrices of variable calcium content were obtained by removing bone matrix from the acid after eight hours. The demineralized bone matrices were washed, freeze dried, and stored at -80°C.

Quantitative determination of calcium

Residual calcium content was then determined using the o-cresolphthalein complexone calcium-binding assay according to the manufacturer's instructions (DMA calcium assay). Briefly, the variably demineralized bone matrices were dissolved in 4.0 ml of 1 N HCl at 90°C overnight. 50 μ L of sample or standard were mixed with 4.0 mL of calcium working

reagent. Calcium working reagent was prepared by mixing equal amounts of calcium color reagent and calcium base reagent. Absorption was measured at 570 nm. Calcium concentrations were calculated from a standard curve using known concentrations of DMA calcium standard. The calcium content was expressed as weight percent calcium of bone matrix dry weight.

Cell line initiation

Initiation of human periosteal cells was accomplished as previously described [19] with some modifications. Periosteum from a girl (eight years old) tibia was obtained during the course of limb amputation with informed consent. The periosteal tissues were washed three times with alpha-minimal essential medium (alpha-MEM, Gibco BRL, Gaithersburg, USA) containing 200 units/mL penicillin and 100 µg/mL streptomycin, cut into small fragments, 1.0 mm x 1.0 mm pieces, and placed with the internal stratum osteogenicum layer facing toward the surface of the T-25 flasks. The preparation was cultured in alpha-MEM supplemented with 10% fetal bovine serum (FBS) and penicillin (100 units/ml)/streptomycin (50 µg/mL) in a 5% CO₂ incubator at 37°C. The outgrowing cells were combined and transferred into T-75 flasks by detachment with a 0.025% trypsin and 0.05% EDTA solution and cultured in the same medium and incubator. When periosteal cells reached confluence, they were split into new T-75 flasks at split ratio 1:4. Cells were continually passaged until sufficient numbers of cells had been generated to provide an opportunity to create a cell bank where cells of a uniform passage number were cryopreserved and stored for use in subsequent studies.

Cell culture

Human periosteal cells were thawed and cultured to sufficient cell numbers and seeded into T-25 flasks at 5.0×10^5 cells per flask (or 2.0×10^4 cells/cm²). These cells were maintained in alpha-MEM supplemented with 10% FBS and penicillin (100 units/mL)/streptomycin (50 µg/mL) until reaching confluence. The alpha-MEM with 10% FBS was then changed to alpha-MEM supplemented with 2% FBS in the absence (as a control) or presence (as an experiment) of either 5 mg SIS, DBM, or SIS/DBM.

Assessment of cell proliferation

Human periosteal cells were plated and treated

under different conditions as described above. After 0, 3, 5, 7, or 10 days of culture, cells were isolated from culture dishes by trypsinization, washed, and cell number and viability were determined with a hemocytometer using trypan blue dye exclusion test. Direct cell counts were performed in duplicate. All experiments were conducted at least twice.

Alkaline phosphatase activity assay

Human periosteal cells were plated in culture and treated as described above. Alkaline phosphatase activity was performed as directed by Wolfenbarger and Zheng [20]. Briefly, treated cells were washed twice with deionized water, scraped with a cell scraper in 3.0 ml deionized water and sonicated at 30% intensity with a cell disrupter for 30 seconds. One milliliter aliquot samples were mixed with 0.2 mL 100 µmole/mL p-nitrophenyl phosphate in 0.15 M 2-amino-2-methyl-1-propanol buffer, pH 10.4, and incubated in a 37°C water bath for 15 minutes. The reaction was stopped by addition of 50 µL 1.0 N NaOH and absorbance at 450 nm was measured. Protein concentrations of the samples were assayed using the BCA protein assay (Pierce, Rockford, USA). The alkaline phosphatase activities were expressed as units of enzyme (nM p-nitrophenyl/min/µg protein).

In vivo bioassay of SIS, DBM, and SIS/DBM composites

The Wistar rat muscle implant model was used to evaluate the osteoinductive potential of the SIS, DBM, and SIS/DBM composites. All experiments were conducted with strict observation of institutional guidelines for the care and use of laboratory animals. The scaffold composites were intramuscularly implanted into Wistar rats to evaluate their *in vivo* bone-forming capability.

Six-week-old male Wistar rats were anesthetized by intra-abdominal injection of sodium penobarbital. After shaving the skin of hind limb, 2 cm incision overlying the posterior aspect of the calf was made under sterile conditions. Muscle pouches were created bilaterally by blunt dissection and subsequently packed with 20 mg of scaffold composite following rehydration with 50 µL of sterile saline solution. Finally, the muscle pouches and skin were closed and the rats were returned to their barrier cages for recovery, housed with food and water, and maintained for 42 days. Histological assessments were carried out at 42 days after implantation to determine the new bone

formation. The explant from each implant site was fixed in 10% formalin, decalcified in 10% formic acid solution, embedded in paraffin, sectioned, and stained with haematoxylin and eosin (H&E) prior to histological evaluations by light microscopy.

Statistical analysis

The data are represented as means with error bars representing standard deviation (SD). Analysis of variance (ANOVA) was used to determine the significant differences among treatment groups. Tukey-type multiple comparison tests were used for comparing means of more than two groups in one way ANOVA analyses. A p-value of less than 0.05 was considered to be statistically significant.

Results

Human periosteal cells were utilized in the *in vitro* cell culture bioassay with SIS, DBM, and SIS/DBM composites. All DBM used in this study contained approximately 2% residual calcium. Our previous studies have documented that DBM with 2% residual calcium yielded high levels of extractable bone morphogenetic protein and provided for maximum osteoinductivity in *in vitro* cell culture-based as well

as *in vivo* animal based bioassays [19, 21].

To examine the proliferation effects of DBM in the *in vitro* assay, the periosteal cells were seeded at 5.0×10^5 cells/T-25 flask in alpha-MEM supplemented with 2% FBS and antibiotics in the presence or absence of 5 mg of SIS, DBM, or SIS/DBM. Cell numbers were counted daily using a cell counter. **Figure 1** demonstrated that SIS, DBM, and SIS/DBM composites tended to promote cell proliferation. Growth of control cells was lower than other groups. On culture-day 5, the cell numbers in SIS/DBM treated cultures sharply increased and became stable on day 7. Growth of cells treated with either SIS or DBM alone was apparently lower than SIS/DBM treated cells but still significantly higher than control on culture-days 5, 7, and 10 ($p < 0.05$).

The mesenchymal cell induction process associated with osteoinductivity of biomaterial typically determined by alkaline phosphatase assay. The alkaline phosphatase (ALP) activity assay was studied with the aim of assessing the effects of composite on osteoblast differentiation of human periosteal cells. Each composite was added into confluent periosteal cell cultures. Human periosteal cells without treatment were used as a control. After

Fig. 1 Proliferation of human periosteal cells cultured for 10 days in the absence and presence of 5 mg SIS, DBM, or SIS/DBM. All data are presented as mean $\pm$ SD.

3, 5, 7, and 10 days of incubation, alkaline phosphatase activity assay was performed. As shown in **Fig. 2**, growth of cells in all groups showed a similar pattern of ALP activity levels. ALP activities of periosteal cells following treatment of each composite gradually increased over culture time. Levels of ALP activity of control, SIS, and DBM were similar throughout the cell-culture period. On culture-day 7, the ALP activity of SIS/DBM treated cells was markedly increased and exhibited the maximal activity level compared to other groups ($p < 0.05$).

The processes of new bone formation in response to stimulation with each composite were visualized by histology assessment. At six weeks post implantation, the results revealed that SIS showed no new bone formation and the implants were surrounded by connective and fibrous tissues (**Fig. 3**). On the contrary, DBM demonstrated new bone formation along the edge of old DBM particles (**Fig. 4**). Most formed cartilage was replaced by the newly formed bone. **Figure 5** illustrated that SIS/DBM composites exhibited high osteoinductivity, osteoblasts secreted osteoid-like matrix and differentiated osteocytes were visible. The residual SIS/DBM composites were surrounded by newly formed bone with osteocytes in lacunae and vascular vessels were visible.

Discussion

Bone tissue engineering has been heralded as an alternative to autografting treatment. A key component of the bone tissue engineering paradigm is a suitable scaffold, which serves as a structural support and delivery vehicle, providing osteoprogenitor cells and bioactive growth factors needed for the formation of new bone tissue [22, 23]. The ideal scaffold should exhibit an environment capable of supporting growing bone tissue and demonstrate good biocompatibility. Moreover, scaffold requirements for bone tissue engineering include osteoinductivity and/or osteoconductivity as well as biodegradability. Several biomaterials have been developed to fulfill these bone tissue engineering requirements [24].

Small intestinal submucosa is an acellular, naturally occurring collagenous extracellular matrix material derived from the submucosa of porcine small intestine that has been shown to contain various bioactive growth factors and cytokines [25]. SIS can provide structural support, and thus stimulates rapid host tissue ingrowth and, ultimately, new bone formation [26, 27]. Despite the good results obtained using SIS, limited studies have investigated the SIS sponge as a biomaterial for bone regeneration. Voytik-harbin and coworkers previously reported that SIS could promote healing of calvaria

Fig. 2 Alkaline phosphatase activity of human periosteal cells cultured for 10 days in the absence and presence of 5 mg SIS, DBM, or SIS/DBM. All data are presented as mean $\pm$ SD.

Fig. 3 Histological evaluation of small intestinal submucosa (SIS) implanted for six weeks. Original magnification, x 100 (a), x 400 (b).

nonunion defects in rats [28]. Furthermore, Suckow et al. [29] demonstrated the ability of SIS to facilitate rapid filling of a surgically created long-bone defect in rats, suggesting that SIS presumably initiate recruitment and seeding of newly formed bone.

Demineralized bone matrix (DBM) represents potential candidates for bone tissue repair because of their close relation in structure and function with autologous bone. Demineralized bone grafts derived from cortical and cancellous bone have been utilized largely for clinical repair of bone injuries in orthopedic, periodontal, and craniomaxillofacial application. DBM acts as a potential biomaterial for use in tissue

engineering because of their ability to support and induce osteogenesis of matrix-incorporated osteoprogenitors. In addition, we recently showed that DBM could induce osteoblast differentiation of mesenchymal stem cells [15, 17] and promoted new bone formation in ectopic site [14]. Accordingly, we used *in vitro* cell culture based and *in vivo* animal based bioassays to evaluate the potential of the SIS/DBM composites to function as the osteoinductive biomaterials for activating new bone formation. The resulting insights may have a substantial impact on the utility of SIS in conjunction with DBM for clinical bone tissue engineering application.

Fig. 4 Histological evaluation of demineralized bone (DBM) implanted for six weeks. Original magnification, x 100 (a), x 400 (b). The newly formed bone is indicated by the arrows.

In our study, SIS/DBM had a tendency to promote more cellular proliferation and osteoblast differentiation than the other groups. Expression of osteoblast phenotypes is sequential. The alkaline phosphatase activity of each group reached the highest level on culture-days 7 and 10. New bone matrix is synthesized and secreted when expression of alkaline phosphatase reaches the highest level to maximize mineralization [30]. The present study also showed that DBM and SIS/DBM had a superior microstructure and supported proliferation and differentiation of osteoblasts while SIS supported only cell proliferation. Therefore, it is conceivable that SIS

in combination with DBM should provide a suitable environment for growth and differentiation of osteoblasts, and eventually enhance new bone formation.

In concordance with our studies, Moore et al. [31] investigated the bone regenerative capability of preformed tubular SIS grafts for bridging segmental bone defects and showed that SIS could not induce new bone formation to fill a critical size segmental bone defect in the rat. Additionally, our observations were consistent with those of other studies indicating that SIS elicits a low-level chronic inflammatory response but that it stimulates exclusively minimal

Fig. 5 Histological evaluation of small intestinal submucosa (SIS)/demineralized bone (DBM) implanted for six weeks. Original magnification, x 100 (a), x 400 (b). The newly formed bone is indicated by the arrows.

fibrous connective tissue deposition [18, 32]. The presence of SIS in the implant site in our animal bioassay is possibly attributed to the relative short period of time (six weeks). The SIS would probably be degradable and replaced with host tissue over time. On the other hand, our results appear to be in contrast to the previous reports of the bone regenerative and osteoinductive capability of SIS [28, 29]. New bone formation throughout the SIS-filled defect and maturing bone with complete healing were observed. The explanation for these discrepancies may be a consequence of differences in material preparation, bioassays applied, or incomplete control for confounding variables. Although SIS did not elicit new bone restoration in this study, the osteoinductive effect was synergistic when DBM was combined with SIS.

Therefore, our findings suggest that SIS/DBM is capable of inducing and conducting new bone formation.

In conclusion, porcine small intestinal submucosa and human demineralized bone represents the interesting and promising potential sources for scaffold-based regeneration and tissue engineering application. These results support the potential to engineer bone by using small intestinal submucosa and demineralized bone materials. The capability of SIS/DBM composites to support the cellular proliferation and differentiation may result in a novel clinical means for bone regeneration therapies as well as provide a new tissue model for *in vitro* studies of osteogenic pathway.

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The authors have no conflict of interest to report.

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