Original

Osteogenic Potential of Demineralized Dentin Matrix as Bone Graft Material

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(Received for publication, February 28, 2017)

Abstract: The aim of this study was to evaluate the osteogenic potential of demineralized dentin matrix (DDM) in supporting the osteogenic activity of MG-63 cells and compare it to a mixture of inorganic bovine bone and collagen (Bio-Oss Collagen®). Cell morphology, differentiation, adhesion, and growth were investigated via scanning electron microscopy and immunofluorescence analyses of F-actin, osteocalcin, and osteonectin. Cell adhesion and growth on the DDM as a bone graft material were more abundant, with cells adopting a flat shape and uniform distribution, than on the Bio-Oss Collagen® at all the observation time points (i.e., 6, 12, 24, 48, 72 h and 14 days). Cell attachment and cytoskeleton organization assessed using confocal laser scanning microscopy revealed that the DDM provided better attachment with cytoplasmic propagation than the control. Immunofluorescence assays showed that the fluorescent intensities of osteocalcin and osteonectin as biomarkers of cellular differentiation were higher on the DDM than on Bio-Oss Collagen® at 6, 12 and 24 h. We showed that the DDM had an enhanced osteogenic potential as a bone substitute for bone augmentation procedures in the dental and medical fields.

Key words: Bone substitutes, Demineralized dentin matrix, Immunofluorescence assay, MG-63 cells, Osteogenic potential

Introduction

Among various bone grafting materials, autogenous bone is considered as the gold standard in terms of osteogenic potential. However, the use of autogenous bone substitutes requires an additional surgical site, which increases morbidity risk1-2.

The bone induction of decalcified dentin implanted with ectopic site and the osteoinductive capacity of bone morphogenetic protein (BMP) was first reported fifty years ago3-5. According to the experiment in 1967, they observed dentin and bone matrix were undergoing similar pattern of reproducible induction system for osteogenesis5. Which results initiated the concept of dentin usage as next choice of bone substitute6-9. Since the development of tooth-derived bone grafting materials, reports showed that the physicochemical characteristics of autogenous demineralized dentin matrix (DDM) were similar to those of autogenous cortical bone9-13. Owing to this advance, bone grafting materials from the autogenous tooth have been used actively, and their clinical applications were approved9-13. Recently, a 5-year-long follow-up of DDM based on cone-beam computed tomography evaluation showed successful guided bone regeneration without complications14. In addition, the first randomized clinical comparison between DDM and inorganic bovine bone (Bio-Oss®) reported that the vertical volume dimension of socket preservation with DDM was as effective as with Bio-Oss®15.

Although there are several reports on the composition (organic versus inorganic substance) and mechanical properties of the DDM, there is little evidence at the cellular level about the osteogenic potential of the DDM and their bone-forming capacity in vitro, such as in human osteoblast-like cells (MG-63 cells)16-18. MG-63 cells were originally isolated from an osteosarcoma cell line, and they displayed many osteoblastic traits that were typical of immature osteoblasts19-21. Therefore, based on their morphology and the ability to produce osteocalcin, a biochemical indicator of bone turnover, MG-63 cells could be a good model for examining the early stages of osteogenic activity22-24.

Bio-Oss Collagen® is used as a bone xenograft material, and is mostly composed of bovine bone mineral that is covered with porcine collagen. The main property of Bio-Oss Collagen® is similar to that of Bio-Oss® in terms of the natural bone mineral of...
The present study was designed to evaluate the osteogenic potential of DDM in terms of supporting osteoblast-like cell growth and osteogenic activity of MG-63 cells and compare it to that of Bio-Oss Collagen®.

Materials and Methods

Teeth collection

Human teeth were collected from the patients who visited the dental clinic in Seoul National University Bundang Hospital. Their teeth were extracted due to orthodontic treatment or non-restorability. This experiment was approved by the Institute of Review Board of Seoul National University Bundang Hospital (B-1412/278-303).

Preparation of DDM from teeth

Extracted teeth were stored in 70% ethyl alcohol in a freezer until they were sent to the Korea tooth bank. The specimen was obtained as DDM, which was similar to the commercially available block-type AutoBT® (AutoBT®: Korea Tooth Bank Co., Seoul, Korea). The DDM was washed, decalcified in a 0.6 N hydrochloric acid, defatted in 1:1 (v/v) chloroform, and lyophilized without being crushed to particles, as previously described.

After this process, the DDM was cut into approximately 50 μm-thick slices using a freezing microtome (Leica CM 3000 Microtome, Bensheim, Germany), fixed on a stained glass slide, and stored in a refrigerator (Fig. 1). Before cell culture on the DDM, the fixatives were removed using sterile distilled water, and the slices were immersed in a solution of 70% alcohol and gentamicin (5 ml alcohol per 0.2 ml gentamicin). The DDM specimens were stored at 2°C until the time of implantation into the culture disc.

Bio-Oss Collagen® (Geistlich, Wolhusen, Switzerland) was used as the control graft material for comparison. Bio-Oss Collagen® is a mixture of 90% Bio-Oss® (inorganic bovine bone; 0.25-1 mm granules) and 10% porcine collagen fibers in a block form. Owing to the similarity in shape of this material to that of DDM, it was prepared in a manner similar to that used for DDM cell culture.

Cell adherence and growth morphology

MG-63 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in α-modified Eagle medium (α-MEM) with 10% fetal bovine serum (FBS), 0.1% hydrocortisone, 1% antibiotic-antimycotic solution, 50 μg/ml ascorbate and 10 mM β-glycerophosphate (Sigma, St Louis, MO, USA). The cultures were incubated in a humidified 5% CO2 incubator at 37°C. The culture medium was changed every 2-3 days.

MG-63 cells were examined at 6, 12, 24, 48, 72 h, and 14 days after culturing on DDM or the control graft material using scanning electron microscopy (SEM; microscope S-4700, HITACHI, Tokyo, Japan). For SEM observations, the cells were fixed using 2.5% glutaraldehyde with PBS for both the DDM and control graft materials.

Immunofluorescence analysis of osteogenic activity

The cells were seeded at a density of 5 × 104 cells/well in 24-well plates on DDM or Bio-Oss Collagen®. After 24 and 48 h of cell culture, the cells were washed with PBS and fixed using a solution of 4% paraformaldehyde. The fixed cells were permeabilized in buffered 0.3% Triton X-100 at room temperature (25°C). The samples were blocked with 1% bovine serum albumin (BSA) for 1 h. The primary antibodies were specific for osteocalcin, osteonectin, or phalloidin (for labeling of F-actin).

In this study, osteocalcin was assessed as a marker of bone formation for osteogenic differentiation. Osteonectin was measured to determine the osteogenic differential activity such as osteoblast formation, maturation, and promotion of crystallization. F-actin was visualized to evaluate the quality of cytoskeleton organization and cell attachment for cell growth. Phalloidin was used for labeling of F-actin.

All the labeled cells were examined using a confocal laser scanning microscope (CLSM) equipped with FITC and DAPI channel filter systems. The immunofluorescence intensities of DDM and Bio-Oss Collagen® were measured at 6, 12, 24, and 48 h for F-actin, osteocalcin, and osteonectin.

Statistical analysis

The immunofluorescence data represented five replicates. The data was statistically evaluated using the Student’s t-test of STATA.
Figure 2. Scanning electron microscopy images.
MG-63 cells were propagated on the demineralized dentin matrix (DDM) and on the control graft material (Bio-Oss Collagen®). In all the observations, cells on the DDM showed more notable morphological changes. (A) At 6 h, MG-63 cells on the DDM showed swollen center of the body. Dentinal tubules were observed (arrow). (B) At 72 h, cells propagated on the DDM with a flat shape and spread out with extended periphery. As a function of time, cells covered the entire surface and extended on the DDM. On the control graft material, the cells appeared rounded and had less extended pods. (C) After 14 days, cells on the DDM showed more flattened shape than those on the control graft material. Multilayered cells were observed on both the DDM and control graft materials.
Results

Cell adherence and growth morphology

Differences in MG-63 cell morphology and propagation between the DDM and Bio-Oss Collagen® were compared at 6, 12, 24, 48, 72 h and 14 days (Fig. 2). Cells that extended on the DDM appeared flatter and spread out over the surface at all stages compared to those on Bio-Oss Collagen®. Cytoplasmic propagations of the MG-63 cells were more evident on DDM than on Bio-Oss Collagen®. At 72 h, MG-63 cells showed elongated morphology with numerous cell to cell contacts on the DDM surface.

By contrast, the MG-63 cells on the Bio-Oss Collagen® were smaller than those on DDM in all the stages. Cells that were seeded on the control were more rounded and less flattened over the surface with fewer numbers of cytoplasmic extensions. At 2 weeks, the cells were multilayered in the DDM, where it showed more vigorous propagation than in the control.

Immunofluorescence analysis for osteogenic activity

F-actin

CLSM images showed F-actin in cells labeled with phalloidin (Fig. 3). Qualitatively, the intensities of fluorescent phalloidin (F-actin) were higher in the DDM group than in the Bio-Oss Collagen® group at 6, 12, 24 and 48 h. Some microfilaments revealed by phalloidin staining were more visible at the cell surface with DDM compared to Bio-Oss Collagen®.

Immunofluorescence analysis for osteocalcin

DNA was stained with DAPI (left panels) and protein was stained via labeling with phalloidin (right panels) as indicated in the Methods. Merged images are shown in the lower panels (×20). (A) Fluorescence intensity was observed in the DDM at 6, 12, 24, and 48 h. (B) Fluorescence intensity was observed in the control at 6, 12, 24, and 48 h. Fluorescence intensity of phalloidin of the actin filament was more obvious in the DDM than in the control at all the stages (6, 12, 24, and 48 h). In the DDM, a clear increase of fluorescence intensity of both cytoplasmic and nuclear signals was observed.

Figure 3. Immunofluorescence analysis of F-actin.

Figure 4. Immunofluorescence analysis of osteocalcin. Osteocalcin showed higher relative fluorescence intensity (a.u.) in the DDM than in the control in 6, 12, and 24 h of observation with statistical significance. At 48 h, the fluorescence intensity in the control was slightly higher than that in DDM, which was not statistically significant. The statistical significance between DDM and the control is indicated by asterisks (* P < 0.05). Arbitrary fluorescence unit (a.u.) represents the mean values of individual fluorescence intensity.
Bio-Oss Collagen activity such as osteoblast-like cell adhesion, growth, potential was enhanced in the DDM in terms of osteogenic cell

In contrast, at 48 h, the fluorescence intensity was higher in the control; however, it was not statistically significant ($P = 0.209$). Arbitrary fluorescence unit (a.u.) represents the mean values of individual fluorescence intensity.

### Osteonectin

Osteonectin showed higher fluorescence intensity at 6 and 24 h in the DDM group than in the Bio-Oss Collagen® group ($P = 0.018$, respectively) (Fig. 4). At 48 h, the fluorescence intensity of the control was higher than that of DDM; however, it was not statistically significant ($P = 0.807$).

### Osteocalcin

Osteocalcin production was measured and compared using immunofluorescence between the DDM and Bio-Oss Collagen® conditions to evaluate differentiation of MG-63. We found that the fluorescence intensity of DDM was significantly higher at 6, 12 and 24 h compared to that of the control ($P = 0.046$, $P = 0.042$, $P = 0.010$, respectively) (Fig. 4). At 48 h, the fluorescence intensity of the control was higher than that of DDM; however, it was not statistically significant ($P = 0.570$).

### Discussion

This study was designed to evaluate whether osteogenic potential was enhanced in the DDM in terms of osteogenic cell activity such as osteoblast-like cell adhesion, growth, differentiation, and maturation.

SEM and immunofluorescence assays showed that the osteogenic potential of MG-63 cells on DDM was higher than that of the control group. Since the physicochemical traits of DDM, such as low crystallinity and solubilities of Ca/P ions, are similar to those of the autogenous cortical bone. Our results may be explained in terms of the inorganic structure and composition of the materials. It is known that the composition of DDM is similar to that of the autogenous bone in terms of the proportion of inorganic (65%) and organic (35%) substances. Experiments, such as the Bradford assay, for assessing the organic composition of DDM suggested that the osteoinductivity of DDM was sufficient for new bone formation. Among the organic components, DDM is composed at 90% of type I collagen and 10% of noncollagenous proteins, including osteocalcin, osteopontin, osterix, Runx2, phosphophoryn, and sialoprotein, which are less represented in Bio-Oss Collagen® and could partially explain the higher osteogenic potential of the DDM in our study.

The SEM images showed that the initial cell attachment and growth of MG-63 cells were favored on the DDM than in the control at all time points. We observed that the intensity of F-actin staining and filament extensions were higher in the DDM, which is consistent with findings based on SEM. The actin cytoskeleton plays a fundamental role in adhesion, which enables cell proliferation, differentiation, maturation, and mineralization during osteogenesis. In this study, increased osteoblast differentiation in DDM may be attributed to higher cell density, which was due to increased cell proliferation.

Osteocalcin is a bone-specific protein released by osteoblasts and is considered as a biochemical indicator of bone remodeling. Osteonectin is considered as a biomarker for the onset of crystal nucleation, and is involved in binding Ca$^{2+}$ ions and hydroxyapatite during mineralization. In concordance with our results, a study reported higher active expression of osteonectin with DDM in a mini-pig cranium defect model at 4 and 12 weeks compared to the control after grafting.

The present study focused only on the initial stages of osteogenesis, i.e., up to 48 h. However, the entire time frame of osteogenesis should also be considered to understand the temporal pattern of expression of osteoblast biomarkers. For instance, a study showed that the differentiation of the osteoblast lineage occurs in two temporal phases: extracellular matrix maturation and extracellular matrix mineralization. Moreover, the authors mention that osteocalcin and osteonectin were upregulated during the maturation phase of differentiation, which started at approximately day 7; therefore, their window of observation was from 3 to 28 days. Similarly, another study observed several osteogenic biomarkers from 4 to 49 days.
comprehend the entire information regarding the alteration in the activities of various biomarkers. This study has a few limitations. Although the Bio-Oss® bone graft material has been more widely used than Bio-Oss Collagen®, the latter was chosen for our study, as we required surface grafting materials and not particulate materials for cell attachment and propagation. Consequently, as collagen comprises 10% of Bio-Oss Collagen®, we were not able to distinguish and measure type I collagen in the control group. However, based on previous studies, the DDM showed favorable results compared to the Bio-Oss® bone graft material in clinical trials. In addition, we did not measure alkaline phosphatase level, which is considered to be the most common bone differential biomarker.

This study provided the basic molecular and cellular evidence of osteogenic activity enhancement in the DDM compared to the control, which has not been documented before. Within the limit of our knowledge, we propose that the DDM possesses osteogenic potential as a bone grafting material for bone augmentation procedures in the dental and medical field. Future research is required to extend the study design to other osteogenic biomarkers and various bone grafting materials.

Acknowledgements
This study was supported by the R & D fund of Seoul National University Bundang Hospital (No. 02-2012-031). The authors are indebted to Professor Emeritus J. Patrick Barron (Tokyo Medical University, Tokyo, Japan, and Adjunct Professor, Seoul National University Bundang Hospital, Seongnam, Republic of Korea) for his pro bono editing of this manuscript.

Conflict of interest
The authors declare no conflict of interests.

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