Microscopic Feature, Protein Marker Expression, and Osteoinductivity of Human Demineralized Dentin Matrix

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Purpose: This study examined the scanning electron microscopic feature, protein marker expression and osteoinductive activity of demineralized dentin matrix (DDM) from human for nude mice.

Materials and Methods: Twenty healthy nude mice, weighing about 20 g were used for study. DDM from Human was prepared and implanted into the dorsal portion of nude mouse. Before implantation, DDM was examined by scanning electron microscopy (SEM). Nude mice were sacrificed at 2 weeks, 4 weeks and 8 weeks after DDM grafting and evaluated histologically by H-E, MT staining. And also immunohistochemistry analysis (ostecalcin, osteopontin) was performed.

Result: Dentinal tubules and collagen fibers were observed by SEM of dentin surface of DDM. The DDM induced bone and cartilage independently in soft tissues. And, the histological findings showed bone forming cells like osteoblasts, fibroblasts at 2, 4 and 8 weeks. On immunohistochemistry analysis, osteocalcin and osteopontin positive bone forming cells were observed.

Conclusion: This results showed that the DDM from human has osteoinductive ability and is a good alternative to autogenous bone graft materials.

Key Words: Demineralized dentin matrix; Immunohistochemistry analysis; Scanning electron microscopy
Bone graft materials include autogenous bone, allogeneic bone, xenogeneic bone, and alloplastic bone, but the most ideal graft material is autogenous bone. Due to the limited amount, inconvenience and aversion of patients including sharp pain after operation, and possibility of postoperative complication, however, research studies on other graft materials that can facilitate bone formation have been conducted.

In the case of xenogeneic bone, it can be affected by a disease, and bone formation ability is much lower compared to autogenous bone. Alloplastic bone graft material reportedly has only osteoinduction ability without osteoconduction, and most cases are caused by “biocompatible filling materials” divided with bony tissue due to the interposition of connective tissue\(^1\). Therefore, based on the many research studies on new bone graft material with bone regeneration ability but without the demerits of allogeneic bone, xenogeneic bone, and alloplastic bone, autogenous tooth bone graft material made by treating the patient’s own tooth was developed. As a material made by extracting bone composition from an extracted autogenous tooth and developed based on the fact that the composition of tooth is almost equal to that of bone\(^2\,3\,5\), autogenous tooth bone graft material provides good biocompatibility without immune or foreign material reaction due to autogenous tissues; it does not cause any adverse reaction of patients in clinical situations, and it is not affected by a disease. In addition, many research studies reported that it can have not only osteoconduction but also osteoinduction abilities\(^6\,9\).

Bone formation is triggered through a complex biological process and controlled by the expression of various bone-related genes. In other words, the continuous expression of bone-related genes affects the bone formation process, which consists of osteoblast proliferation, bone matrix formation, and calcification\(^10\,14\).

In the osteocyte differentiation stage, collagen and alkaline phosphatase are expressed at the early stage, and osteopontin and osteocalcin, at the later stage\(^19\). As the most useful bone formation marker currently used in clinical situations, osteocalcin is assumed to contribute to bone mineralization and is also a very unique protein for bone. Thus, evaluating the activity of osteoblast and examining the level of bone formation are deemed very useful\(^16\).

Osteopontin reportedly attaches osteoblast to the extracellular matrix during the early stage of bone formation and adjusts the size of growing crystals by combining with them during bone calcification\(^17\). Therefore, in immunohistochemical stain, the expression of these proteins may be a marker of bone regeneration with hard tissue formation ability\(^18\).

The chemical compositions of tooth and alveolar bone are very similar. Dentin and alveolar bone have almost identical composition, with the dentin of human tooth containing many bone growth factors including type I collagen and bone formation-inducing protein. In particular, in 1 mm\(^2\) are 20,000~50,000 dentinal tubules with diameter of 0.9~2.5 \(\mu\)m\(^18\,20\).

Although the fact that the osteoinduction-stimulating agent in dentin is bone morphogenic protein (BMP) is not proven at the molecular level, growth factors facilitating bone formation are confirmed to exist, such as transforming growth factor-\(\beta\), basic fibroblast growth factor, and insulin-like growth factor\(^21\,22\).

This study sought to examine the expression of protein marker and bone formation ability based on the histological examination of the osteoinduction ability of demineralized dentin matrix (DDM) and immunohistochemical analysis of osteocalcin and osteopontin by manufacturing DDM from extracted human tooth, analyzing it histologically, and then transplanting under the skin of nude mouse.
Materials and Methods

1. Materials

1) Test Animals
A total of 20 nude mice (weight: about 290 g) from Orient Bio Co., Ltd. (Seongnam, Korea), were used as test animals. Normal mice after 5 days of adaptation period were used. This test observed the policies of the Korean Association for Laboratory Animal Science and laws related to animal test.

2) Manufacture of Human DDM
The extracted teeth were put in 70% ethyl alcohol and delivered to a specialized treatment agency (Korea Tooth Bank Co., Seoul, Korea) to remove foreign materials such as adhering soft tissues and tartar. Each tooth was then divided into crown and root and ground. Particles ground into 1–2 mm were put in distilled water and hydrogen peroxide and washed with ultrasonic washer to remove foreign materials. The washed particles were dehydrated with ethyl alcohol and defatted with ethyl ether solution. Particles were used for transplant following lyophylization and ethylene oxide gas sterilization.

2. Methods

1) Scanning Electron Microscopic Analysis of Graft Material
To examine the surface of DDM used in the test, a scanning electron microscope (SEM) (S-4800; Hitachi, Tokyo, Japan) was used. The surface of specimen was coated with 7 nm platinum and examined under x5,000 and x10,000 magnifications.

2) Transplant Under the Skin of Nude Mice
Pentobarbital sodium (43 mg/kg, Nembutal, Dainabot Co., Tokyo, Japan) was injected into the abdominal cavity of nude mice for general anesthesia. The surgical site was then sterilized and isolated. The dorsal portion of nude mice was cut, and pouches were created under the skin of nude mice on both sides. DDM was then transplanted into the pouch.

3) Manufacture and Examination of Tissue Specimen
The test animals were sacrificed 2, 4, and 8 weeks after the transplant to collect DDM and

![Dental tubule, Dense Collagen](image)

**Fig. 2.** Scanning electron microscope micrograph of dentin surface of demineralized dentin matrix (Original magnification, left: ×5,000, right: ×10,000).

![Cell number](image)

**Fig. 1.** Morphometry of bone forming cells.
adjacent tissues, and the collected tissues were put in 10% buffered formalin for 10 days and subsequently demineralized using formic acid. Specimens were manufactured, and H&E and MT stain were performed. The specimens were examined using an optical microscope. After

Fig. 3. (A) Histologic finding of demineralized dentin matrix (DDM) after 2 weeks (H&E staining, ×100): highly vascularized surrounding fibrous tissues (black arrow). (B) Histologic finding of DDM after 2 weeks (H&E staining, ×500): dense fibrous tissues with moderate blood vessels and newly attached fibroblast are observed around dentin particles (white arrows). (C) Histologic finding of DDM after 2 weeks (H&E staining, ×200): well encapsulated dentin particles with in cells supplies and cellular activities on the surface of dentin which seems to be a activation of fibroblasts are observed (white arrow). (D) Histologic finding of DDM after 2 weeks (H&E staining, ×500): newly attached cells are observed around dentin particles (white arrow). Activated cells produced new collagen on dentin surface: osteoid (black arrow). (E) Histologic finding of DDM after 2 weeks (MT staining, ×200): activated cells on the surface of dentin looked like an osteoblast (black arrow). Also produced newly deposited collagens which we can call “Osteoid” (white arrow). (F) Histologic finding of DDM after 2 weeks (H&E staining, ×200): see the cell activation into osteoblasts and newly produced collagen matrix which were embedded with osteocytes (black arrows).
that, immunohistochemical stain on osteopontin and osteocalcin was performed using a primary antibody.

4) Immunohistochemical Analysis
The level and ratio of immunohistochemical stain were divided into 4 grades. In particular, the level of stain was divided into no-stain, mild, moderate,

Fig. 4. (A) Histologic finding of demineralized dentin matrix (DDM) after 4 weeks (H&E staining, ×100): ① Well encapsulated dentin matrix and well organized fibrous tissues between the particles. ② Fibrous tissue is an reservoir of angiogenesis as well as stem cell supplies (black arrows). (B) Histologic finding of DDM after 4 weeks (H&E staining, ×500): ① Newly deposited and produced collagen matrix on dentin particles. ② Still activated osteoblast like cells on the surface of dentin. ③ Some vacuoles in the newly formed osteoid seems to be a osteocytes (black arrows). (C) Histologic finding of DDM after 4 weeks (H&E staining, ×200): ① Osteoblastic activity from surrounding fibroblasts are profound. ② Dense fibrous tissues turns into highly vasculated tissues (black arrows). (D) Histologic finding of DDM after 8 weeks (H&E staining, ×500): surround fibrous tissues are changed into highly vascularized tissues (white arrows). (E) Histologic finding of DDM after 4 weeks (H&E staining, ×500): features of new bone formation from surrounding fibrous tissues which are the mesenchymal stem cell's supplies as well as vasculaities (black arrows). (F) Histologic finding of DDM after 4 weeks (H&E staining, ×200): dense field phenomenon of new bone formation with random osteocyte embedding (black arrows).
and severe, with 0, 1, 2, and 3 points assigned, respectively. The ratio of stain was divided into no-stain, 10–30%, 30–60%, and more than 60%, and 0, 1, 2, and 3 points were assigned, respectively. The product of each stain’s level and ratio was then assigned as the total score (Fig. 1).

![Histologic finding of demineralized dentin matrix (DDM) after 8 weeks (H&E staining, ×200): more activated osteoblastic activities are observed (black arrow).](image1)

![Histologic finding of DDM after 8 weeks (MT staining, ×200): More activated osteoblastic layers surrounding dentin particles.](image2)

![Histologic finding of DDM after 8 weeks (MT staining, ×100): see the blue staining as newly formed collagen (black arrow).](image3)

![Histologic finding of DDM after 8 weeks (H&E staining, ×100): newly formed osteoid surrounding dentin particles spreads to the whole graft area where the dentin particle disappeared completely (black arrows).](image4)

![Histologic finding of DDM after 8 weeks (H&E staining, left: ×100, right: ×500): induced cartilage and bone marrow by dentin matrix are observed (black arrows).](image5)

![Histologic finding of DDM after 8 weeks (H&E staining, ×500): induced bone formation (osteoid, woven bones) maturated to lamellar bone which has basic four environmental units such as periosteum, lamellar bone, endosteum, and bone marrow (black arrows).](image6)

Fig. 5. (A) Histologic finding of demineralized dentin matrix (DDM) after 8 weeks (H&E staining, ×200): more activated osteoblastic activities are observed (black arrow). (B) Histologic finding of DDM after 8 weeks (MT staining, ×200): ① More activated osteoblastic layers surrounding dentin particles. ② Looks like osteoblastic cell band surrounded dentin particles (black arrows). (C) Histologic finding of DDM after 8 weeks (MT staining, ×100): see the blue staining as newly formed collagen (black arrow). (D) Histologic finding of DDM after 8 weeks (H&E staining, ×100): newly formed osteoid surrounding dentin particles spreads to the whole graft area where the dentin particle disappeared completely (black arrows). (E) Histologic finding of DDM after 8 weeks (H&E staining, left: ×100, right: ×500): induced cartilage and bone marrow by dentin matrix are observed (black arrows). (F) Histologic finding of DDM after 8 weeks (H&E staining, ×500): induced bone formation (osteoid, woven bones) maturated to lamellar bone which has basic four environmental units such as periosteum, lamellar bone, endosteum, and bone marrow (black arrows).
Result

1. Scanning Microscopic Examination

To examine the surface of DDM, a SEM (S-4800; Hitachi) was used before transplanting into nude mice. Dentinal tubules and dense collagen matrix were identified in the demineralized dentin, and collagen matrix was expressed clearly around the dentinal tubules (Fig. 2).

2. Histological Analysis

In the 2nd week, fibroblast and blood vessel were examined in the soft tissues around the dentin particle (Fig. 3A, 3B). Moreover, there was a dentin particle covered with thin fibrous capsule (Fig. 3C, 3D). Osteoid was created around the newly attached osteoblast, and there was a newly created collagen matrix around the osteocyte (Fig. 3E, 3F).

In the 4th week, fibrous tissues between dentin particles were developed, and there was collagen matrix around the particles (Fig. 4A, 4B). In addition, vascularized tissues were expressed clearly, and osteocyte was covered with new bone around the bone graft material (Fig. 4C, 4D). New bone was generated on one side (Fig. 4E, 4F).

In the 8th week, a more developed osteoblast was examined, and the collagen group and bony tissue were calcified gradually. Osteoinduction - a phenomenon wherein new bony tissues are generated around DDM particles - was identified (Fig. 5A~D). Cartilage connecting the DDM particles like a bridge was formed (Fig. 5E); the bone matrix had lamellae expressed in mature bony tissue caused by a decrease of osteoid and trabecular bone (Fig. 5F).

3. Result of Immunohistochemical Stain

In the 2nd, 4th, and 8th weeks, the marker (osteocalcin and osteopontin) was expressed in the immunohistochemical stain. Osteocalcin and osteopontin were expressed more at the marginal location of dentin particle and in all cells, including fibroblast around the dentin particle where new bone is formed actively, osteoblast, and osteocyte. Note, however, that the clearest expression was in the fibroblast (Figs. 6, 7).

In the immunohistochemical stain of osteocalcin and osteopontin, the score of the osteocalcin group in the 2nd, 4th, and 8th weeks is 9, 8, and 8, respectively, and that of the osteopontin group is 6,
Discussion

Many research studies on the osteoinduction ability of bone graft material reported that not only the chemical composition but also a specific surface type - including microporous surface structure of graft material - are important in order for the graft material to have ectopic bone formation. Since mesenchymal cells such as osteoblast and osteocyte measure 10–20 μm but osteoclast is 20~100 μm, the latter cannot infiltrate into dentinal tubules. When examining demineralized dentin with SEM before transplanting into a nude mouse, however, organic matter around the dentinal tubules was found to have been preserved; the bone formation materials included in the organic matter were also assumed to induce the formation of new bone from soft tissues. In addition, due to their unique microporous structure, dentinal tubules can be a good bone graft material that increases the absorption and invasion of growth factors affecting bone formation.

In 1970, Huggins et al. reported the cartilage and osteoinduction of dentin matrix derived from pig, mouse, and nude mouse. In 1986, Inoue et al. investigated the osteoinduction of nude mouse’s DDM.

Reddi searched the osteoinduction ability of demineralized bone matrix and non-demineralized bone matrix and reported that demineralization increases osteoinduction ability. According to Yasaku, inorganic matter in dentin interferes with

Fig. 7. Osteopontin immunohistochemistry of demineralized dentin matrix: arrows indicate osteopontin positive bone forming cells (bone specific sialo-protein I).

Fig. 8. Morphometry of immunohistochemistry (cell number).
osteoinduction, and demineralization is important in the expression of osteoinduction ability. In the case of demineralized dentin, bone formation protein such as BMP is reportedly attached to the matrix continuously\(^ {30}\), antigenicity decreases because inorganic matter and cell composition are removed, and increase of surface area occurs due to the exposed dentinal tubules\(^ {31}\). In the existing research on tooth ash wherein organic matter is completely removed due to a possible transmission of infection and disease\(^ {32}\), the regeneration of alveolar bone was limited because organic matter with osteoinduction ability was removed. Note, however, that the current manufacturing method of DDM can have osteoconduction ability derived from the microstructure of dentin and osteoinduction ability derived from preserved organic matter by preserving both organic and inorganic matter.

In this study, athymic mice without immune reaction were used. During the test, partial formation of cartilage and lamellated bone was identified. Moreover, in the 2nd, 4th, and 8th weeks, there were bone formation cells around the transplanted DDM. The DDM particle area was absorbed, and new bone and lamellated bone were formed on one side; such can be regarded as osteoinduction. In the histomorphometric analysis, the number of bone formation cells increased in the 4th week compared to the 2nd week but decreased in the 8th week compared to the 4th week. This may have been caused by BMP, which operates at the early stage of bone formation.

With this study, based on the immunohistochemical examination of human DDM transplanted in soft tissues, the expression of protein marker can be said to be related to bone formation and calcification such as osteopontin. Osteocalcin can be regarded as a good tool for identifying human DDM’s osteoinduction ability and potential ability to form hard tissues.

Note, however, that this study has limitations because the statistical significance of the histomorphometric analysis cannot be verified and scoring of stain level in the immunohistochemical examination has low objectivity. We will compensate for such limitations and continue research.

**Conclusion**

DDM was transplanted under the skin of nude mice, and its osteoinduction ability was evaluated with a histological method as well as immunohistochemical analysis. In analyzing human DDM with a SEM, dentinal tubules and collagen were identified, and cartilage and bone were induced independently within soft tissues under the skin of nude mice.

**References**


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