Journal of Hard Tissue Biology 15[1] (2006) p6-10
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CODEN-JHTBFF, ISSN 1341-7649

Original

Histopathological Study of Matrix Mineralization by Osteoblastic-like and Odontoblastic-like Cells in Diffusion Chamber

Andrea Paola Rodriguez[1)], Silvia Borkosky[1)], Phuu Pwint Han[1)], Hidetsugu Tsujigiwa[1)], Tohru Takagi[1)], Sathi Gulsan Ara[1)], You-Jin Lee[1)], Naoki Katase[1)], Mika Okauchi[1)], Keisuke Nakano[1)] and Jing Xiao[1,2)]

[1)] *Department of Oral Pathology and Medicine, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 2-5-1 Shikatacho, Okayama 700-8525, Japan*
[2)] *Dalian Medical University, Faculty of Dentistry, China*
(Accepted for publication, September 18, 2005)

Abstract:Bone and dentin resemble each other in composition and mechanism of formation and mineralization. Preliminary analysis of osteoblastic and odontoblastic cells before using them in tissue engineering is mandatory. Thus, in order to evaluate the process of calcification, we evaluated osteoblastic-like (KUSA/A1cells) and odontoblastic-like (MDPC-23 cells) cells seeded in cell culture and in intraperitoneal diffusion chamber. Our results indicated that KUSA/ A1 cells differentiated into osteoblasts-like cells and induced bone tissue inside the chamber. Whereas, MDPC-23 cells showed odontoblastic phenotype without ability to induce dentin formation, suggesting that MDPC-23 cells are special cells, which lost the capacity to induce mineralized dentin matrix after long period of time.This study showed the significance of basic information of calcification process by osteoblastic-like and odontoblastic-like cells before using them in tissue engineering.

Keywords: Diffusion chamber, KUSA/A1 cells, MDPC-23 cells, TEM, Von Kossa staining.

Introduction

Bone and dentin resemble each other in composition and mechanism of formation and mineralization[1)]. In the formation of bone and dentin, terminally differentiated osteoblasts and odontoblasts secrete an extracelullar matrix that mineralizes outside the cells[2)]. These mineralized tissues are similar in many respect: they both consist principally of collagen fibrils, primarily of type I collagen with carbonate apatite crystals within and around the fibrils[2)]. Tissue engineering is the biomedical technology to build a suitable environment utilizing cells, scaffold, growth factors, or appropriate combination of the three[3)]. Because of this, we believe that preliminary analysis of the cells to induce calcified bone and dentin matrix before using them in tissue engineering is mandatory.

In this study, we used KUSA/A1 and MDPC-23 cell line. KUSA/A1 cells are bone marrow stromal cell line from primary bone marrow culture of female C3H/He mice[4)]. These cells combined with atelocollagen scaffold are capable to induce new bone formation in vivo[5)]. MDPC-23 cells are mouse dental papillae cell line derived from 18-day CD-1 fetal mouse[6)].

In order to evaluate the process of calcification in vitro and in vivo, osteoblastic-like (KUSA/A1cells) and odontoblastic-like (MDPC-23 cells) cells were seeded in cell culture and intraperitoneal diffusion chamber.

Corresponding Author: Andrea P Rodriguez, Department of Oral Pathology and Medicine, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, Okayama, Japan.2-5-1 Shikata-cho, Okayama city 700-8525, Japan,Phone (+81) 86-235-6652, Fax (+81) 86-235-6654, E-mail: andrearodriz@yahoo.com

Materials and Methods

Animals

For in vivo experiment, we used 4-week-old male severe combined imunodeficient (SCID) mice.This study was performed in accordance with the Guidelines for Animal Experiments of Okayama University Medical School, Japanese Government Animal Protection and Management Law (No.105) and Japanese Government Notification on Feeding and Safekeeping of Animals (No.6).

Cell culture

KUSA/A1 cells were courtesy of Dr. A. Umezawa from Keio University, Tokyo, Japan and MDPC-23 cells were courtesy of Dr. C.T. Hanks from University of Michigan. The cells were cultured in minimum essential medium alpha medium (α-medium, GIBCO BRL, Inc., USA) supplemented with 10% fetal bovine serum (SIGMA, USA) and 1% antibiotic-antimycotic (GIBCO, USA). Then, they were seeded in 10 cm petridishes (Falcon, Inc., USA) and incubated at 37ºC in humid air with 5% CO2.

In order to induce calcification in vitro, the cells were exposed to Ascorbic acid (AA, 50 μg/ml) and β-glycerophosphate (β-GP 6 mM). The growth medium was changed every 3 days until the

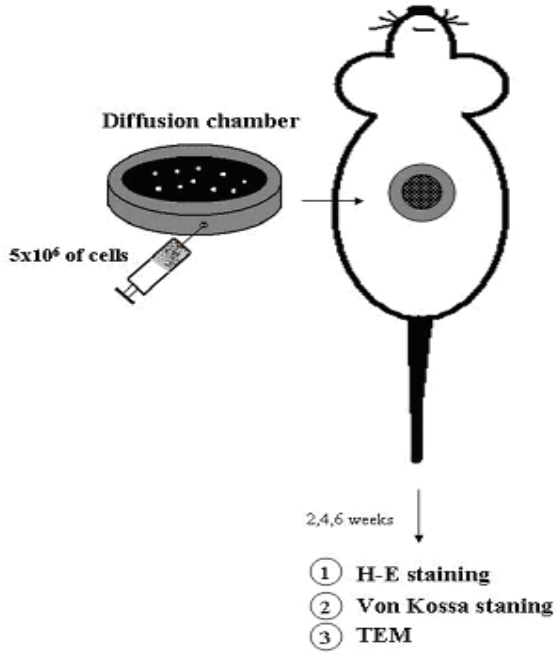

Fig.1.Scheme of in *vivo* experimental design. The cells were seeded in diffusion chambers and implanted in the peritoneal cavity. The animals were sacrificed at 2, 4 and 6 weeks after implantation. The implants were subjected to H-E staining, Von Kossa staining and TEM.

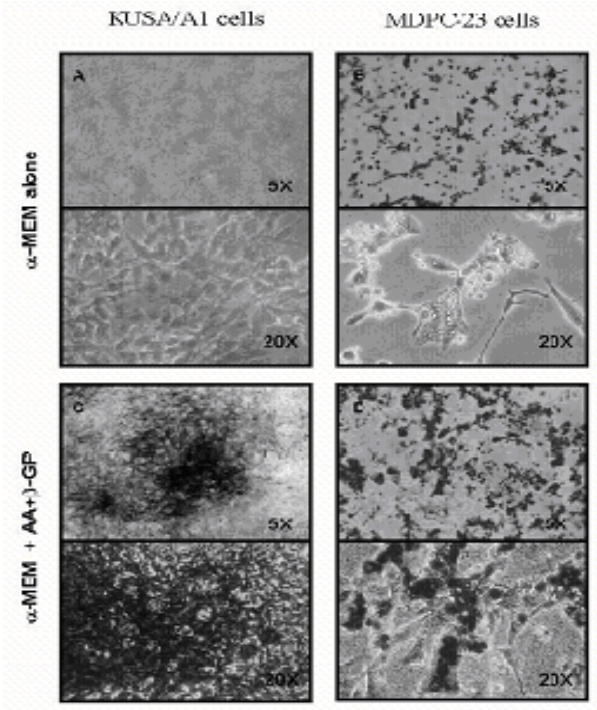

Fig.2.Von Kossa staining of KUSA/A1 and MDPC-23 cells on 14 days in vitro study. A) KUSA/A1 cells cultured in α-MEM alone did not show evidence of calcification. B) Degenerative and apoptotic MDPC-23 cells seeded in α-MEM alone were noted. C) Calcium deposition in bone matrix was observed in KUSA/A1 cells exposed to AA+ β-GP. D) Dystrophic calcification was demonstrated in MDPC-23 cells exposed to AA+ β-GP.

cells were nearly confluent. The cells were analyzed at 1, 2, 3, 7, 10 and 14 days.

Peritoneum implantation and explantation

Twelve SCID mice were subjected to intramuscular anesthesia with Nembutal (Dianabot, USA). The ventral surface of the skin was shaved, disinfected with 70% alcohol and iodine and cut by blunt dissection to form peritoneum pockets. $5x10^6$ of KUSA/A1 cells and $5x10^6$ MDPC-23 cells were placed in 6 diffusion chambers respectively, and implanted in the peritoneum. The animals were sacrificed with an overdose of ether at 2, 4 and 6 weeks after implantation. The chambers were removed, fixed with 4% paraformadehyde, embedded in paraffin, sectioned at 4 μm in thickness, and stained with routine hematoxylin and eosin, Von Kossa staining and also subjected to transmission electron microscopy (Fig.1).

Transmission electron microscopy (TEM)

After 2,4 and 6 weeks, the chambers were fixed with 2% glutataraldehyde and 2% PFA buffer with a pH of 7.4 for 2 hours at 4 ºC, postfixed in 2% OsO4 buffer with pH of 7.4 for 2 hours at 4 ºC, then dehydrated in ethanol series and embeded in EPON 812. Ultrathin sections were cut with a diamond knife using Reichert Ultracute Microtome (Germany). The sections were stained with 2% uranyl acetate and nitrate and examined using a H-800 TEM.

Results

1. In vitro analysis

KUSA/A1 cells exposed to AA+ β-GP showed small areas with calcium deposition on 3 days, whereas large and diffuse mineralized areas were observed at 7, 10 and 14 days (Fig.2C). MDPC-23 cells exposed to AA+ β-GP revealed few calcium depositions at 1 day and more deposition at 3 days. However, these odontoblastic-like cells demonstrated strong degeneration with calcium depositions at 7, 10 and 14 days(Fig.2D). Control groups of KUSA/A1 and MDPC-23 cells cultured in α-MEM alone did not showed evidence of calcification even at 14 days (Fig.2A, 2B).

2. Histological analysis

KUSA/A1 cells at 2 weeks revealed early immature bone formation attached to the membrane of the chamber. Osteoid formation, osteocytes, and active osteoblasts were observed. Matrix protein secretion was also observed within the porous of the membrane (Fig.3A). KUSA/A1 cells at 4 weeks showed more amount of bone with many osteocytes within the lacuna formation, osteoid and mineralized matrix. Matrix proteins were also detected within the porous of the membrane (Fig.3B). KUSA/A1 cells at

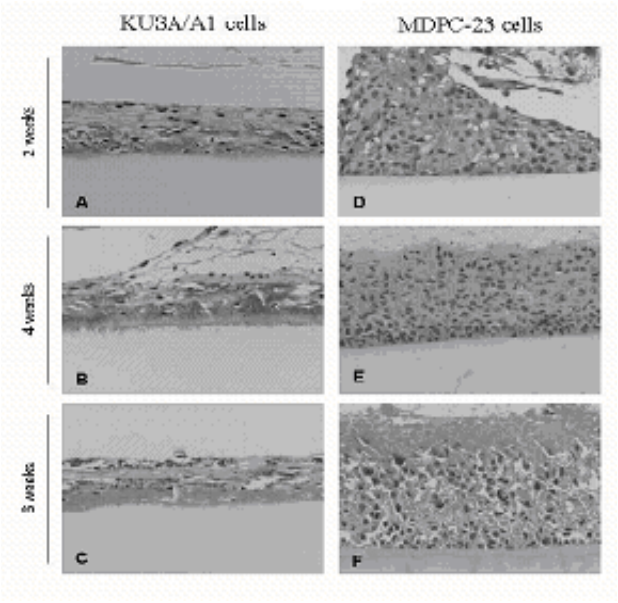

Fig.3. Histological examination of KUSA/A1 (A, B, C) and MDPC-23 (D, E, F) cells in vivo study. H&E, 80X. A) At 2 weeks, early immature bone formation occurred within the chamber. B) At 4 weeks, large amount of new bone with osteocytes within the lacunae and secreted proteins inside the porous of the chamber membrane were observed. C) The presence of degenerative cells and bone tissues at 6 weeks. D) Excessive cellular proliferation at 2 weeks, E) irregular polarized odontoblast-like cells at 4 weeks, and F) degenerative and apoptotic cells at 6 weeks were detected.

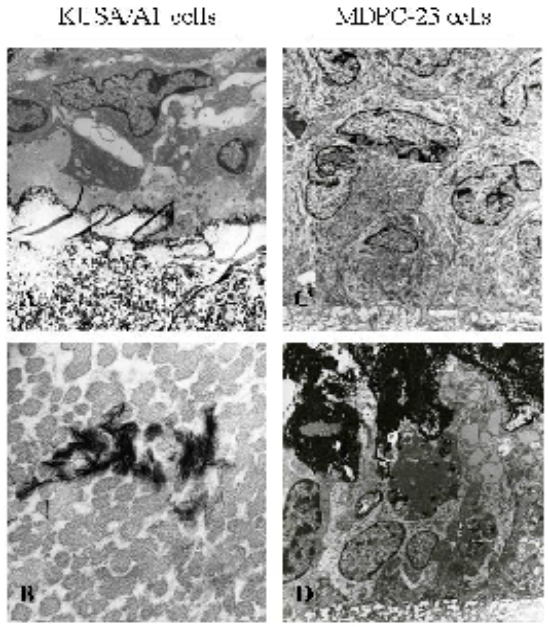

Fig.5. Transmission electron microscopy of KUSA/A1 and MDPC-23 cells. A) Irregular osteoblast-like cells producing extracellular bone matrix and calcium deposition inside the membrane of the chamber can be observed. B) Immature collagen fibers with initial calcification produced by KUSA/A1 cells are also seen. C) Polarized columnar odontoblast-like cells and D) dystrophic calcification in the opposite side of the membrane are present.

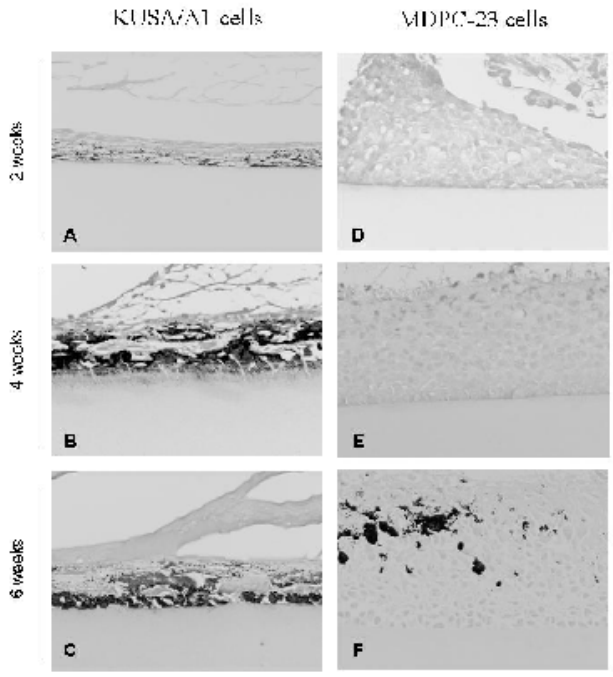

Fig.4.Calcification examination of KUSA/A1 (A, B, C) and MDPC-23 (D, E, F) cells in vivo study. Von Kossa staining, 80X. A,B,C) In KUSA/A1 cells at 2,4 and 6 weeks, calcium deposition in the bone matrix were shown. D,E) In MDPC-23 cells at 2 and 4 weeks, slightly calcium deposition was revealed into the cytoplasm. F) Presence of large areas of dystrophic calcification was demonstrated in MDPC-23 cells at 6 weeks.

6 weeks revealed degenerative cells and bone tissue (Fig.3C). MDPC-23 at 2 weeks showed excessive cellular proliferation. The cells were seen attached to membrane of the chamber (Fig.3D). MDPC-23 at 4weeks demonstrated the presence of polarized cuboidal and columnar cells similar to odontoblasts. Although matrix formation was not detected (Fig.3E). At 6 weeks, the cells became degenerative and apoptotic (Fig.3F).

3. Calcification analysis (Von Kossa staining)

KUSA/A1 cells at 2, 4 and 6 weeks showed evidence of calcium deposition within the bone matrix stained by Von Kossa (Fig.4A, B, C). In the case of MDPC-23, slight calcium deposition within the cytoplasm was noted at 2 and 4 (Fig.4D, E). However, there were large areas of dystrophic calcification at the opposite side of the membrane at 6 weeks (Fig.4F).

4. Transmission electron microscopy

KUSA/A1 cells at 2 weeks revealed extracellular matrix produced by irregular osteoblast-like cells, mineralized bone attached to the membrane of the chamber and calcium deposition within the membrane (Fig.5A). The initial calcification started on the collagen fibers (Fig.5B). MDPC-23 cells at 6 weeks showed presence of polarized odontoblast-like cells and large areas of dystrophic calcification at the opposite side of the membrane (Fig.

5C, D).

Discussion

Bone and dentin resemble each other in composition mechanism of formation and mineralization[1]. At the beginning of matrix mineralization, the mineral crystals grow within defined cellular or extracelullar spaces, but the crystals have random orientations and crystal habits comparable to those of inorganically grown crystals[7]. After that, matrix-mediated mineralization, the crystals have well-defined orientations relative to the matrix, they have narrow size ranges, and they adopt unique crystal habits[8,9]. Mineralization processes in bone, dentin and cementum are the matrix-mediated type. Moreover, it has been reported that extracellular matrix of the mineralized tissues contains three groups of components[10,11]. The structural matrix macromolecules define the shape and structure of the mineralizing compartment. In the bone, dentin and cementum, this matrix is the type I collagen fiber network. Second, there is a group of structural protein-interactive, mineral phase-interactive acidic matrix proteins. In the bone and dentin, they are mainly phosphoproteins. Finally, there is a third group of modifiers, enzymes that can degrade or otherwise modify the acidic matrix proteins during the mineralization process[10,11].

The basic premises are that the acidic matrix proteins are delivered into the ECM after formation of the structural protein framework and that the acidic proteins then interact specifically with the structural proteins[7]. It is further proposed that the matrix-associated acidic proteins determine the locus of crystal deposition and regulate nucleation of mineralization[7]. Interactions between the nucleated crystal and other nonmatrix-associated acid proteins may regulate crystal growth and determine crystal size and shape[7].

It has been reported that Ascorbic acid is required for the synthesis of collagen, the regulation of alkaline phosphatase activities and protein synthesis in cultures of osteoblasts-like cells[12]. The organic phosphate, β-glycerophosphate (β-GP), has been used in vitro to provide a potential source of phosphate ions[12]. In order to study matrix mineralization in vitro, the cells were exposed to α-MEM+AA + β-GP and α-MEM alone as control. MDPC-23 and KUSA/A1 cells placed in α-MEM alone did not show calcified areas. However, when MDPC-23 cells were exposed to AA+ β-GP, they demonstrated intracytoplasmic calcium deposit on day 1 and increased the intracytoplasmic calcium deposits on 3 days. MDPC-23 cells showed deposit of calcium salts in degenerating and dead cells without any evidence of matrix formation on 7, 10 and 14 day. In contrast, KUSA/A1 cells show diffuse calcified areas on 3, 7, 10 and 14 days. These results suggest that MDPC-23 revealed dystrophic calcification and KUSA/A 1 cells showed evidence of calcium deposition after bone matrix formation.

In order to analyze the process of calcification in vivo, Odontoblast-like and osteoblast-like cells were seeded in diffusion chamber and implanted in intraperitonial cavity. KUSA/A1 cells differentiated into osteoblast-like cells and induce mineralized bone formation in vitro and in vivo, whereas MDPC-23 cells showed odontoblastic phenotype without the ability to induce dentin formation.

In normal bone formation, initial calcification occurs in or around matrix vesicles[13]. Ectopic bone formation induced by BMP-collagen complex has demonstrated multiple nucleation sites: matrix vesicles and carrier collagen fiber[14]. Moreover, it was described by Nagai et. al.[15] that carrier alone revealed mineralizing foci on fine collagen fibrils, which plays a role as the site of the bone in nucleation and calcification. Similar results were observed in our study by TEM: KUSA/A1 cells showed evidence of calcium deposition within the bone matrix and initial calcification on the collagen fibers. On the other hand, MDPC-23 cells revealed the presence of large areas of dystrophic calcification on the opposite side of the membrane. This result indicates the presence of dystrophic calcification in MDPC-23 cells by deposits of calcium in areas with necrotic cells for lack of oxygen and nutrition supply.

In summary, our in vitro and vivo results demonstrated that KUSA/A1 cells are capable of differentiating into osteoblast-like cells and induce mineralized bone matrix, whereas MDPC-23 cells are special cells, which have morphological phenotype of differentiated odontoblast without mineralized dentin matrix induction. This could be due to two possibilities: absence of odontogenic epithelium for reciprocal interaction in cell culture and diffusion chamber; or aging and sensibility of MDPC-23 cell line.

This study showed the significance of basic information of calcification process by odontoblastic-line and osteoblastic-like cells before using them in tissue engineering.

Acknowledgement

This study was supported by Grant-in-Aid for Scientific Research (A) from the Japanese Ministry of Education, Culture,Sports, Science and Technology (No. 1520960). Also, we would like to thank Mr. Tadao Zohda for his expert technical support in TEM.

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