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Influence of the microenvironment on gene and protein expression of odontogenic-like and osteogenic-like cells

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Key words: MDPC-23 cells, KUSA/A1 cells, diffusion chamber, RT-PCR analysis, immunohistochemical analysis

ABSTRACT: Progenitor cells play an important biological role in tooth and bone formation, and previous analyses during bone and dentine induction have indicated that they may be a good alternative for tissue engineering. Thus, to clarify the influence of the microenvironment on protein and gene expression, MDPC-23 cells (mouse dental papilla cell line) and KUSA/A1 cells (bone marrow stromal cell line) were used, both *in vitro* cell culture and in intra-abdominal diffusion chambers implanted in 4-week-old male immunodefficient mice (SCID mice). Our results indicate that KUSA/A1 cells differentiated into osteoblast-like cells and induced bone tissue inside the chamber, whereas, MDPC-23 showed odontoblast-like characteristics but with a low ability to induce dentin formation. This study shows that MDPC-23 cells are especial cells, which possess morphological and functional characteristics of odontoblast-like cells expressing dentin sialophosphoprotein *in vivo*. In contrast, dentin sialophosphoprotein gene and protein expression was not detected in both cell lines *in vitro*. The intra-abdominal diffusion chamber appears as an interesting experimental model for studying phenotypic expression of dental pulp cells *in vivo*.

Introduction

Tissue regeneration is a biological process for renewing damaged tissue. In order to stimulate regeneration, different kinds of biomaterials such as porous apatite, poly DL-lactic-co-glycolic acid scaffold, porous beta-tricalcium phosphate, L-lactide/e-caprolactone copolymer foams, calcium alginate, CO_3apatite-collagen sponge can be used (Kasai *et al*., 2000; Abukawa *et al*., 2003; Watanabe *et al*., 2004; Bo *et al*., 2003; Nakao *et al*., 2003; Shang *et al*., 2001; Tieliewuhan *et al*., 2004). Tissue engineering is the biomedical technology to build a suitable environment, utilizing cells, scaffolds, growth factors, or appropriate combination of the three (Tabaka, 2003). We believe that preliminary analysis of cell phenotypes before using them in tissue engineering is mandatory.

In this study, we compared MDPC-23 and KUSA/A1 cell lines. KUSA/A1 cells are bone marrow stromal cells from primary bone marrow culture of female C3H/He mice (Umezawa *et al*., 1992). These cells are capable of expressing three mesenchymal phenotypes, namely, osteocytes, adipocytes and myotubes, after treatment with 5-azacytidine during cell culture (Umezawa *et al*., 1992). On their part, MDPC-23 cells are mouse dental papilla cell line derived from 18-day CD-1 fetal mouse (Hanks *et al*., 1998). These cells are epithelioid in shape with multiple cell membrane processes and capable of expressing dentin sialoprotein and dentin phosphoprotein in culture (Hanks *et al*., 1998).

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The use of diffusion chambers with membranes impermeable to cells, and which offers an experimental condition permitting only the free passage of molecules present in the humoral phase of the host, has been reported (Niskanen *et al*., 1989). Osteogenic cells are present in the bone marrow stroma of animals including rodents and human, and their ability to produce bone-like mineralized tissue has been demonstrated in diffusion chambers loaded with bone marrow cells (Ochi *et al*., 2003; Friedesntein *et al*., 1987). However, epithelial-mesenchymal interaction is necessary to induce normal tooth development. In cell culture, i. e., in absence of the enamel organ; the environmental conditions found in intact living tissues in situ are not mimicked. Because of this, we speculated that dental pulp cells would be capable of expressing dentin-specific genes and proteins inside the diffusion chamber in the host microenvironment.

Thus, to clarify the influence of the microenvironment in protein and gene expression, MDPC-23 cells (mouse dental papilla cell line) and KUSA/A1cells (bone marrow stromal cell line) were seeded either in cell culture or in intra-abdominal diffusion chambers.

Materials and Methods

Cell culture

MDPC-23 cells were a courtesy of Dr. C. T. Hanks (University of Michigan) and KUSA/A1 cells were a courtesy of Dr. A. Umezawa (Keio University, Tokyo, Japan). The cells were cultured in alpha-minimum essential medium (α-MEM, 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 Petri dishes (Falcon, Inc., USA) and incubated at 37ºC in humid air with 5% CO_2. The growth medium was changed every 3 days until the cells were nearly confluent.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted using TRIzol (Life Technologies, Inc., USA) from MDPC-23 and KUSA/A1cultured in α-MEM during 1, 4, 7, 10 and 14 days (Fig. 1A). Mouse dental pulp, used as positive control, was obtained from the mandibles of C57BL6 mice. After excision, the mandibles were frozen, crashed, and the total RNA was extracted using TRIzol according to the manufacturer's instruction. The extracted and purified total RNA was treated with RNase-free DNase I (Takara, Inc., Japan) to remove the contaminating DNA. The final total RNA was dissolved in diethylpyrocarbonate-treated water and the absorption at 260 nm and 280 nm was measured by a spectrophotometer (DU-640, Beckman Instruments Inc., USA) to calculate both quantity and purity. Complementary DNA was synthesized using the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Life Science, Inc., USA). To synthesize cDNA, 0.5 µl of oligo d(T) primer 12-18 mer (0.5 µg/l) was added to 5 µg of total RNA.

Quantitative RT-PCR was performed using a thermocycler (PC707, Astec, Inc., Japan). Then, 25 mM $MgCl_2$ and specific primer for each gene (1.5 mM) was added to 1 µl of cDNA reaction mixture obtaining a final concentration of 0.25 µM. Then 1.25 U of Platinum Taq DNA polymerase (Life Technologies, Inc., USA) was added and the final volume was adjusted to 25 µl. The thermal cycling condition for DNA amplification of dentin sialophosphoprotein and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, as a control "housekeeping gene") was set at 94ºC for 30 sec, 58ºC for 30 sec and 72ºC for 30 sec.

A fixed quantity of the amplified product was sampled at several amplification cycles (18 to 30 cycles), electrophoresed on agarose gel, and stained with ethidium bromide. The target amplification products were compared under the condition that the PCR amplified products did not reach a saturated state. The base sequences of the primers used for the amplification of dentin sialophosphoprotein were (F) 5'-CGACCCTTGTCCAGGA-3' and (R) 5'-CATGGACTCGTCATCGAA-3', while those for glyceraldehydes 3-phosphate dehydrogenase were (F) 5'-GGGTGGAGCCAAACGGGTC-3' and (R) 5'-GGA

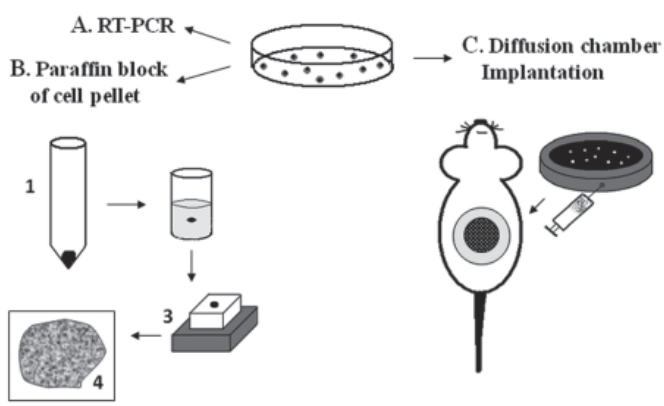

FIGURE 1. Overview of experimental procedures.

GTTGCTGTTCAAGTCGCT-3' as a control "housekeeping gene".

Paraffin block of cultured cell pellet

After 1, 4, 7, 10 and 14 days of cell culture, the cells were separated from the culture dishes using a scraper and centrifuged at 1500 rpm for 10 minutes. Then, the cell pellets were transferred to a plastic vial, fixed with 4% paraformadehyde, embedded in paraffin, sectioned at 4 μm and stained. Hematoxylin-eosin was used for routine histology; immunostaining is described below (Fig. 1B).

Animals

Four-week old, genetically deficient male mice (severe combined immunodeficiency, SCID) were used. 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).

Diffusion chamber implantation

Twelve SCID mice were subjected to peritoneal anesthesia with 40 mg/kg sodium pentobarbitone (Dianabot, USA). The ventral surface of the skin was shaved and disinfected with 70% ethanol and iodine. The skin was cut and the abdominal cavity was opened by blunt dissection, and a single diffusion chamber was implanted underneath the liver, containing either $5x10^6$ of KUSA/A1 cells, as a control, or $5x10^6$ MDPC-23 cells (Fig. 1C). 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 (see below).

Immunostaining for dentin sialophosphoprotein, type I collagen, osteocalcin and osteonectin

Polyclonal antibodies against dentin sialophosphoprotein (kindly provided by Dr. C. T. Butler), type I collagen (LSL, Japan), osteocalcin (LSL, Japan) and osteonectin (LSL, Japan) were employed for immunostaining by the peroxidase anti-peroxidase method (DAKO, Denmark) in the following steps: 1) inactivation of endogenous peroxidase by hydrogen peroxide in methanol for 30 minutes; 2) microwave treatment before blocking nonspecific protein binding by swine normal serum (DAKO) containing 1% bovine serum albumin for 10 minutes; 3) incubation with the primary antibodies was made at 4ºC overnight and at the following dilutions: 1:100 for dentin sialophosphoprotein, 1:500 for type I collagen, 1:100 for osteocalcin and 1:500 for osteonectin, containing 1% bovine serum albumin; 4) incubation with anti-rabbit IgG (DAKO) at a 1:40 dilution for 30 minutes; 5) incubation with peroxidase/antiperoxidase complex at a dilution of 1:40 for 30 minutes; 6) treatment with diaminobenzidine and counterstaining with Mayer's hematoxylin.

Immunostaining for osteopontin

The sections were immunostained with monoclonal antibodies against osteopontin (IBL, Japan) using Vectastain Avidin-Biotin-peroxidase Complex Mouse Kit method (ABC, USA). The main steps were as follows: 1) inactivation of endogenous peroxidase by hydrogen peroxide in methanol for 30 minutes; 2) the activation of antigenicity was done by microwave treatment before blocking nonspecific protein binding by rabbit normal serum and horse normal serum respectively for 10 minutes at room temperature; 3) incubation with the primary antibody at 4ºC overnight. The optimal dilution of osteopontin was 1:50; 4) incubation by anti-mouse IgG (1:200), respectively for 30 minutes; 5) incubation with the avidin-biotin-peroxidase complex at a dilution of 1:50 for 30 minutes; 6) treatment with

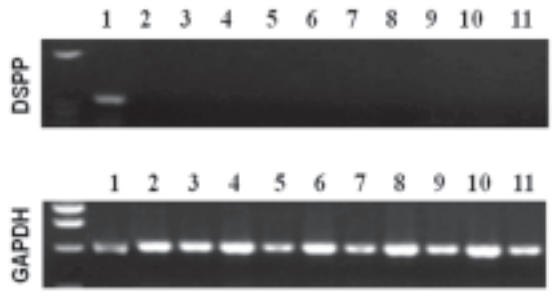

FIGURE 2. Agarose gel electrophoresis of RT-PCR amplification for dentin sialophosphoprotein (DSPP) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Lane 1 is mouse dental pulp; lanes 2-6 are KUSA/A1 cells on days 1, 4, 7, 10 and 14; lanes 7-11 are MDPC-23 cells on days 1, 4, 7, 10 and 14. While the dental pulp expressed both genes in culture, neither KUSA/A1 cells nor MDPC-23 cells expressed DSPP.

diaminobenzidine and counterstaining with Mayer's hematoxylin.

Results

1. RT-PCR analysis

The total mRNA was extracted on culture days 1, 4, 7, 10 and 14 from MDPC-23 and KUSA/A1 cells grown in α-MEM. Dentin sialophosphoprotein expression was negative in KUSA/A1 and MDPC-23 cells (Fig. 2).

2. Cell pellets analysis

KUSA/A1 cells cultured in α-medium showed cords of elongated cells (Fig. 3A). MDPC-23 cells cultured in α-MEM formed a homogenous epithelioid mass (Fig. 3C). Moreover, cells cultured in α-MEM presented cellular degeneration after 10 days. The immunostaining for dentin sialophosphoprotein was negative in KUSA/A1 and MDPC-23 cells (Fig. 3B,D).

3. Histological analysis of implants in the diffusion chamber

KUSA/A1 cells at 2 weeks revealed early immature bone formation attached to the chamber's membrane. Osteoid formation, osteocytes, and active osteoblasts were observed. Matrix protein secretion was also observed within membrane pores (Fig. 4A). KUSA/A1 cells at 4 weeks showed greater degree of bone formation, and both osteoid tissue and a mineralized matrix. Many osteocytes within their lacunae and protein secretion within membrane pores were also detected (Fig. 4B). KUSA/A1 cells at 6 weeks revealed degenerative cells and bone tissue (Fig. 4C). MDPC-23 at 2 weeks showed a marked cellular proliferation. The cells were seen attached to the chamber's membrane (Fig. 4D). MDPC-23 at 4 weeks showed both polarized cuboidal and columnar cells similar to odontoblasts (Fig. 4E).

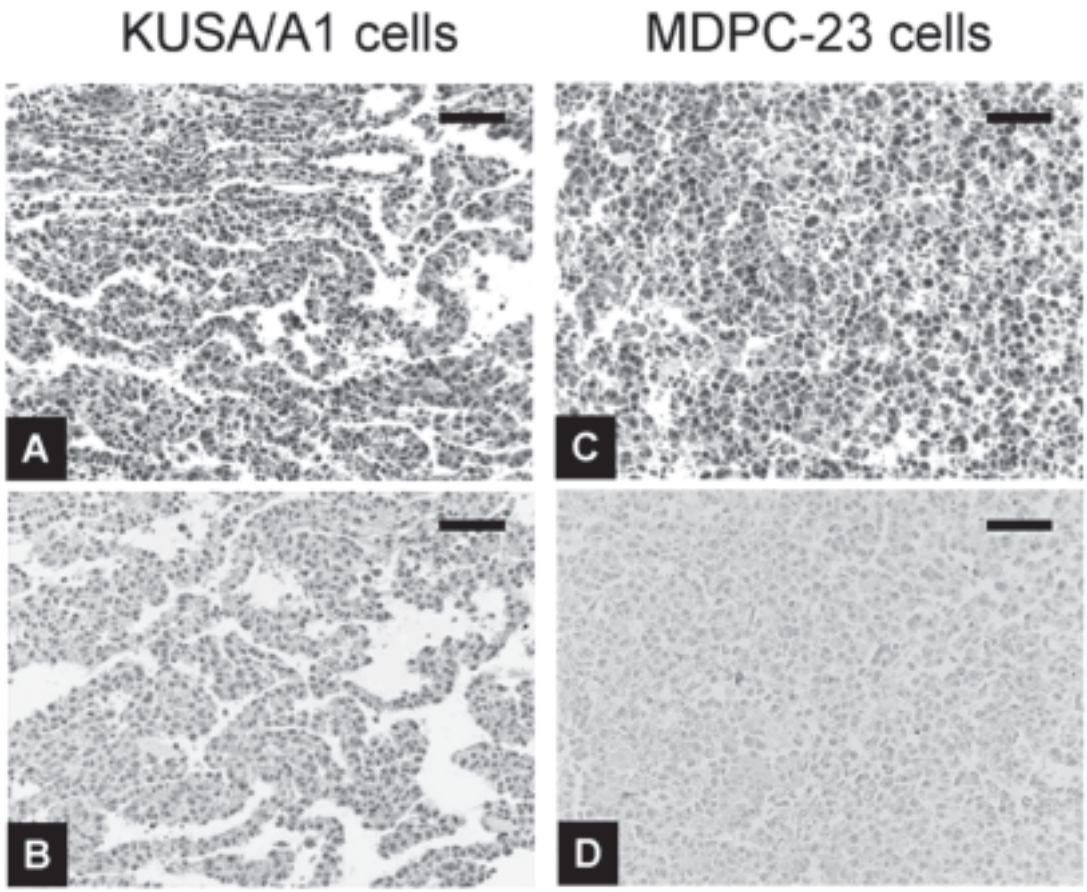

FIGURE 3. Dentin sialophosphoprotein expression (B-D) and hematoxylin-eosin staining (A-C) of KUSA/A1 and MDPC-23 cells on culture day 7. **A.** KUSA/A1 cells forming cords of elongated cells. **B.** KUSA/A1 cells cultured in α- MEM showing negative reaction for dentin sialophosphoprotein. **C.** MDPC-23 cells forming a homogeneous epithelioid mass. **D.** MDPC-23 cells cultured in α- MEM showing negative reaction for dentin sialophosphoprotein. Bar = 100 μm

MDPC-23 at 6 weeks revealed the presence of degenerative odontoblast-like cells (Fig. 4F).

4. Immunohistochemical analysis of implants in the diffusion chamber

Localization of dentin sialophosphoprotein (Fig. 5)

Dentin sialophosphoprotein was completely negative in KUSA/A1 cells at 2, 4 and 6 weeks. In contrast, dentin sialophosphoprotein were strongly expressed within the cytoplasm of MDPC-23 cells, especially in those attaching the chamber's membrane at 2 and 4 weeks. At 6 weeks, it was observed within the membranes of diffusion chamber in MDPC-23 cells.

Localization of type I collagen (Fig. 6)

Bone formation was observed in implants of KUSA/A1 cells at 2, 4 and 6 weeks. Type I collagen was detected in osteoid, mineralized bone matrix and differentiated osteoblasts. Type I collagen was also de-

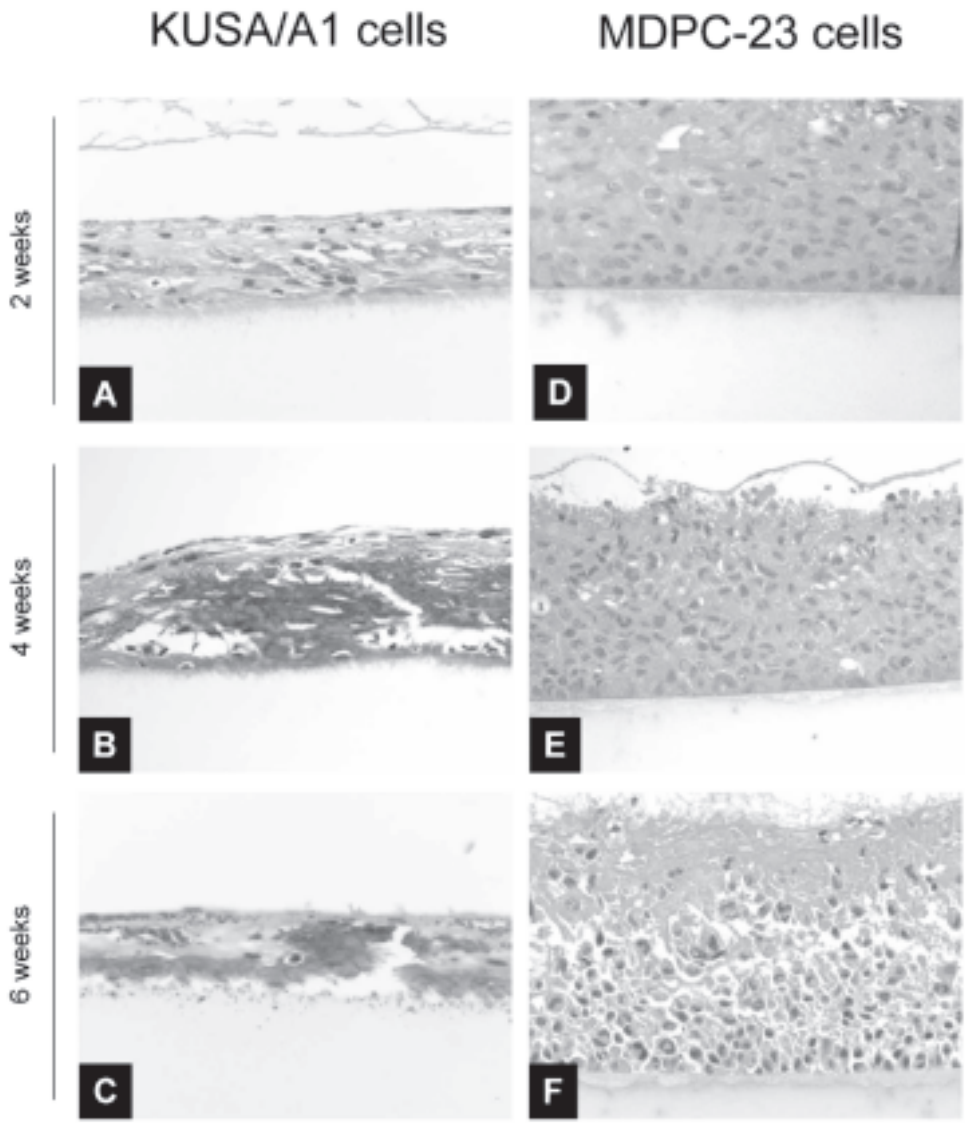

FIGURE 4. Histological examination of KUSA/A1 and MDPC-23 cells in the diffusion chamber. **A.** KUSA/A1 cells showing early immature bone formation after 2 weeks. **B.** KUSA/A1 cells showing new bone formation and osteocytes within the lacunae, as well as protein secretion through the membrane pores after 4 weeks in the chamber. **C.** KUSA/A1 cells showing degenerative cells and bone tissues after 6 weeks in the chamber. **D.** Massive proliferation of MDPC-23 after 2 weeks. **E.** MDPC-23 cells have generated irregularly polarized odontoblast-like cells after 4 weeks. **F.** MDPC-23 cells showing degeneration and apoptosis after 6 weeks. Hematoxylin-Eosin, Bar = 50 μm.

tected in implants of MDPC-23 cells at 2 weeks but immunostaining was very weak. Immunostaining was distributed not only in the cytoplasm of the MDPC-23 cells but also in intercellular areas at 4 weeks. At 6 weeks, type I collagen was clearly detected in odontoblast-like cells and within the chamber's membrane.

Localization of osteopontin (Fig. 6)

The specimens were positively immunostained for osteopontin particularly in front of the newly mineralized bone induced by KUSA/A1 cells at 2, 4 and 6 weeks. Osteopontin expression was also detected in MDPC-23 cells and within the chamber's membrane at 2, 4 and 6 weeks.

Localization of osteocalcin (Fig. 6)

Osteocalcin-positive reactions were clearly observed in osteoblasts, bone matrix and within the chamber's membrane in KUSA/A1 cells at 2, 4 and 6 weeks. In contrast, osteocalcin was seen in a few

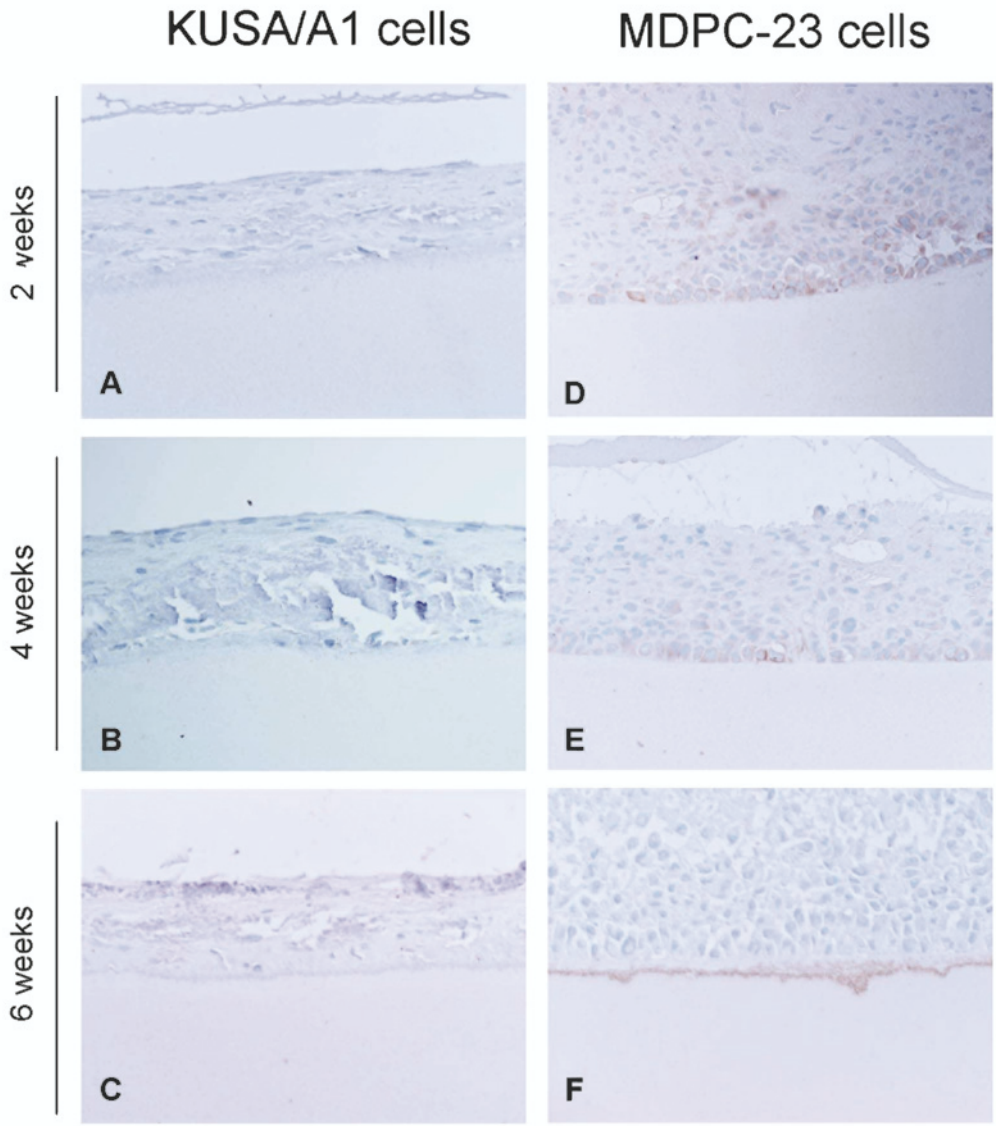

FIGURE 5. Expression of dentin sialophosphoprotein by KUSA/A1 and MDPC-23 cells in the diffusion chamber. A, B and C. Negative immunostaining for dentin sialophosphoprotein in KUSA/A1 cells at 2, 4 and 6 weeks after implantation in diffusion chamber. D and E. Positive immunostaining for dentin sialophosphoprotein in the cytoplasm of MDPC-23 cells, especially in those attaching to the chamber's membrane, after 2 and 4 weeks. F. MDPC-23 cells 6 weeks after implantation in diffusion chamber, immunostaining has spread to the membrane. Bar = 50 μm.

MDPC-23 cells at 2 weeks, but strongly positive at 4 and 6 weeks.

Localization of osteonectin (Fig. 6)

Osteonectin expression was very weak in KUSA/A1 and MDPC-23 cells at 2 weeks, but it was strongly positive, especially in the cytoplasm, at 4 and 6 weeks.

Discussion

Both bone and dentin resemble each other in their composition and mechanism of formation (Qin *et al*., 2002). In addition to major levels of type I collagen, both tissues contain non-collagenous proteins that play important biological roles in osteogenesis and dentinogenesis (Qin *et al*., 2002).

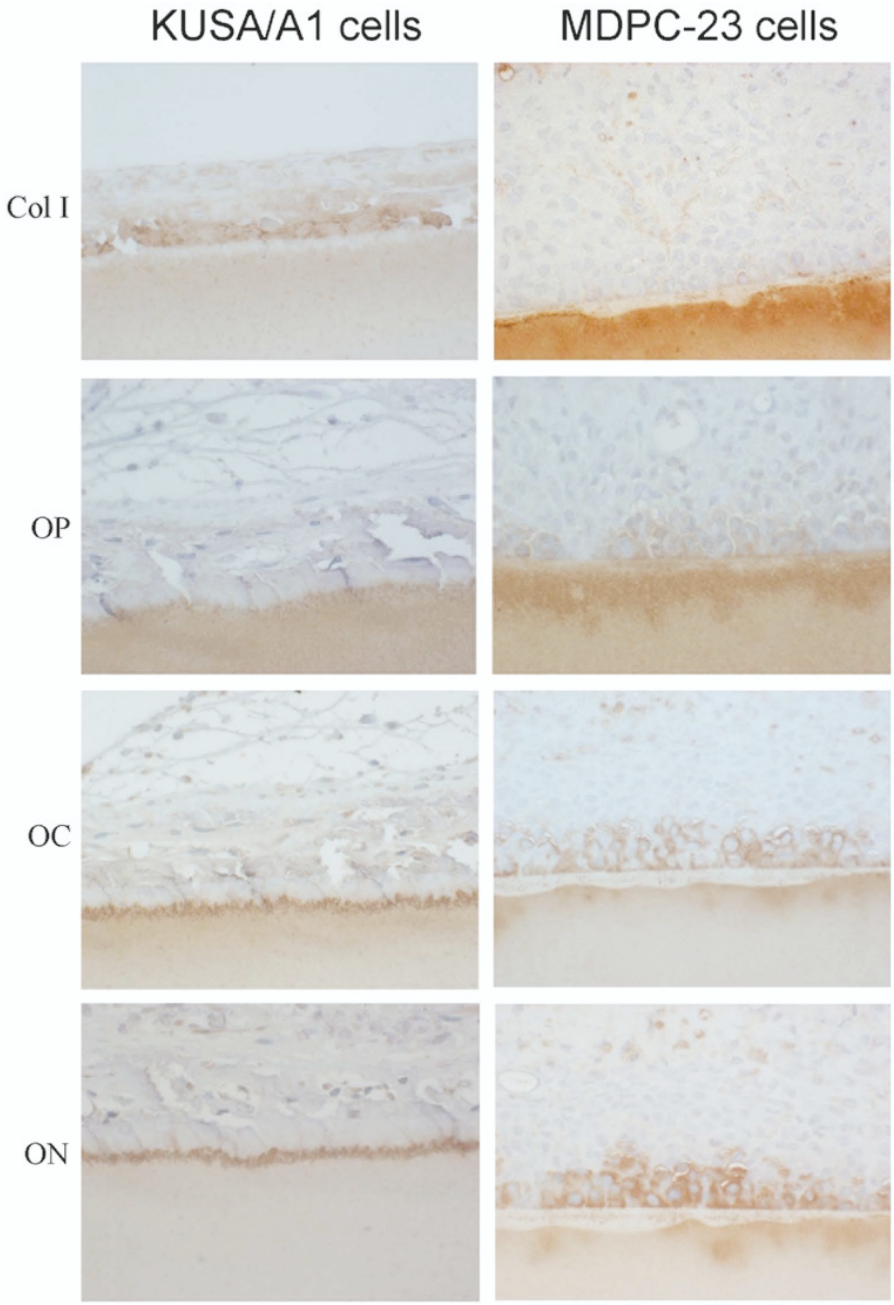

FIGURE 6. Immunohistochemical analysis of KUSA/A1 and MDPC-23 cells in the diffusion chamber at 4 weeks after implantation. Positive immunolocalization for type I collagen (Col I), osteopontin (OP), osteocalcin (OC) and osteonectin (ON) were detected in both cell lines. Bar = 50 μm.

During tooth organogenesis, type I collagen is primarily expressed in polarized odontoblasts, followed by the osteocalcin gene expression (Bleicher *et al*., 1999). Concomitantly, polarized ameloblasts start to accumulate amelogenin mRNAs and transiently express the dentin sialophosphoprotein gene (Bleicher *et al*., 1999). At the same time, osteocalcin gene expression decreases in secretory odontoblasts (Bleicher *et al*., 1999). Osteocalcin may thus act as an inhibitor of mineralization whereas dentin sialoprotein/phosphophoryn would be involved in more advanced steps of mineralization (Bleicher *et al*., 1999). The transient expression of dentin sialophosphoprotein in ameloblasts suggests a role of epithelial-mesenchymal interactions in the formation of the tooth (Bleicher *et al*., 1999; Butler *et al*., 1997). MDPC-23 cells are epithelioid in shape and are capable of synthesizing and secreting dentin sialoprotein and dentin phosphoprotein during cell culture, suggesting that this cell line is from the odontoblastic lineage (Hanks *et al*., 1998). However, in our study, dentin sialophosphoprotein was not detected in MDPC-23 cells *in vitro* (Figs. 2 and 3D). This may be due to the absence of the enamel organ in cell culture, thus *in vitro* method cannot mimic the environmental conditions found in intact living tissues *in vivo*.

Because of this, we believe that MDPC-23 cells are capable of expressing dentin sialophosphoprotein within a host microenviroment as we have shown by using an implanted diffusion chamber. Diffusion chamber's membranes are impermeable to cells, offering an experimental condition in which only free passage of molecules in the humoral phase of the host is permitted (Niskanen *et al*., 1989). Both bone and cartilage formation have been reported for bone marrow stromal cells *in vivo* diffusion chambers (Ashton *et al*., 1980; Gundle *et al*., 1995), and this has validated the diffusion chamber as an experimental system to study human osteogenesis using appropriate cell populations (Gundle *et al*., 1995). In this study, we used both KUSA/A1 and MDPC-23 cells placed in diffusion chambers. The KUSA/A1 cells were capable of inducing new bone-like tissue, which were immunopositive for type I collagen, osteocalcin, osteonectin and osteopontin (Fig. 6). The MDPC-23 cells expressed type I collagen, osteocalcin, osteonectin, osteopontin and dentin sialophosphoprotein (Figs. 5 and 6). These results suggested that KUSA/A1 and MDPC-23 cells are able to express markers for both osteoblasts and odontoblasts differentiation in a diffusion chamber.

Dentin sialophosphoprotein has been reported to be present in dentin at high levels and also in bone at very low levels (Qin *et al*., 2002). Another immunohistochemical study revealed that dentin sialoprotein was synthesized by both young and mature odontoblasts, as well as by dental pulp cells and preameloblasts, but not by osteoblasts, chondrocytes or other cell types (Butler *et al*., 1997). Our experiments are in agreement with the latter study in that the expression of dentin sialophosphoprotein by odontoblast-like cells of MDPC-23 transplants was high but no expression was detected in either osteoblast-like cells or bone matrix of KUSA/A1 cells transplants.

In vivo odontoblasts are polarized columnar cells which synthesize the dentin matrix (Hanks *et al*., 1998). Some other odontoblast cell lines such as RPC-C2A (Kasugai *et al*., 1988) and RDP4-1 (Kawase *et al*., 1990) from adult male rat incisor pulp cells were not capable of synthesizing dentin-specific proteins (MacDougall *et al*., 1995). In the present study, the MDPC-23 cells near the diffusion chamber's membrane were polarized columnar cells, often ovoid or low (Figs. 4D and E) and expressed dentin sialophosphoprotein (Figs. 5D, E and F), suggesting that a signaling pathway is involved for the expression of dentin sialophosphoprotein in MDPC-23 cells *in vivo*, which is distinct from the usual dental epithelial-mesenchymal interaction in normal tooth development. It has been reported that macrophage inflammatory protein-3alpha (MIP-3α) and beta-defensin-2 (βD-2) stimulate dentin sialophosphoprotein gene expression in human pulp cells (Shiba *et al*., 2003). Interestingly, these genes are expressed in different organs of the peritoneal cavity, such as small intestine, liver, etc. We believe that MIP-3α and βD-2 might stimulate the dentin sialophosphoprotein expression of MDPC-23 cells in intra-abdominal cavity. The next step of this study would be isolation of the specific growth factor to stimulate dentin formation. In order to develop a good restorative material for caries treatment, this growth factor could be combined with various current dentins bonding system following the experimental model indicated by Harnirattisai (Harnirattisai and Hosoda, 1991).

In summary, KUSA/A1 cells were capable of differentiating into osteoblast-like cells and induce bone formation inside the chamber, whereas MDPC-23 cells are special cells, which have a morphological and functional phenotype of differentiated odontoblasts, expressing dentin sialophosphoprotein only *in vivo*. This study suggests that the diffusion chamber is a good alternative to analyze gene and protein expression of dental pulp cells *in vivo*. This experimental model could be considered as an "*in vivo* cell culture method" for studying cell phenotypic expression in basic research.

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