In vitro differentiation of human dental follicle cells with dexamethasone and insulin

C. Morsczeck a,*, C. Moehl a, W. Götz b, A. Heredia c, T.E. Schäffer c, N. Eckstein a, C. Sippel a, K.H. Hoffmann a

a Stiftung caesar, Center of Advanced European Studies and Research, Ludwig Erhard Allee 2, 53175 Bonn, Germany
b Rheinische Friedrich-Wilhelms-Universität Bonn, Zentrum für Zahn-, Mund- und Kieferheilkunde Poliklinik für Kieferorthopädie Oralbiologische Grundlagenforschung Welschnonnenstr 17, 53111 Bonn, Germany
c Westfälische Wilhelms-Universität Münster, Center for Nanotechnology (CeNTech) and Physikalisches Institut, Gievenbecker Weg 11, 48149 Münster, Germany

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Abstract

The dental follicle is an ectomesenchymally derived connective tissue harboring precursor cells for the tooth supporting apparatus. In this study, we examined gene expression of freshly isolated human dental follicle cells during osteogenic differentiation in vitro. These plastic adherent fibroblastic cells express Notch-1, nestin and vimentin. We differentiated dental follicle cells with dexamethasone or insulin-based protocols into membrane-like structures containing mineralizing foci. An analysis of mineralized tissue with atomic force microscopy illustrated a bone and cementum-like structure. A real-time RT-PCR analysis was developed to investigate expression of typical osteoblast or cementoblast related genes during differentiation. Gene expressions of osteocalcin (OCN), bone morphogenic protein (BMP)-2 and nestin were increased during the both differentiation approaches. Our work demonstrates differentiation of dental follicle cells with an insulin-based protocol for the first time.

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1. Introduction

The dental follicle is a loose ectomesenchymally derived connective tissue surrounding the enamel organ and the dental papilla of the developing tooth germ prior to eruption. One important biological function of the dental follicle is the coordination of teeth eruption. The dental follicle harbors progenitor cells for the periodontium, the supporting apparatus of the tooth (Ten Cate et al., 2003). The periodontium is composed of the periodontal ligament (PDL), the alveolar bone and the mineralized bone-like cementum. Recently, progenitor cells were identified in dental follicles of bovine tooth germs released by digestion with bacterial collagenase (Handa et al., 2002a, 2002b). The differentiation capacity of these follicle cells was proven by in vivo tests with SCID mice. Here, cells formed a cementum-like matrix as opposed to bovine PDL fibroblasts or bovine alveolar osteoblasts (Handa et al., 2002a, 2002b). Seo et al. (2004) isolated human PDL stem cells from a pooled cell population of different PDLs of third molars. Under defined culture conditions, these cells differentiated into cementoblast-like cells. Recently, we isolated dental follicle precursor cells that demonstrated osteogenic differentiation in vitro and in vivo (Morsczeck et al., 2005). These cells express Notch-1 and nestin. In
tooth development, gene expression of Notch is regulated by epithelial–mesenchymal interactions and is associated with determination of ameloblast cell fate (Mitsiadis et al., 1995). Nestin is an intermediate filament associated with undifferentiated neural progenitor cells and odon-to-blasts of the dental pulp (Lendahl et al., 1990; Terling et al., 1995; About et al., 2000).

Studies have shown that it is possible for mesenchymal cell populations to contain two uniquely different categories of undifferentiated cells: progenitor cells that are committed to a particular lineage, and multipotent stem cells that are not committed to any particular lineage (Rogers et al., 1995; Young et al., 1999). The insulin/dexamethasone analysis compares and contrasts the separate effects of insulin and dexamethasone to identify the cells in question. Insulin will accelerate phenotype in progenitor cells but will have no effect on multipotent cells. By contrast, dexamethasone will induce lineage commitment and expression of phenotype in multipotent cells. Osteogenic differentiation was demonstrated with dexamethasone and insulin-based protocols by Young et al. (1999).

In this study, we investigated gene expression of osteogenic differentiation markers and precursor cell markers in human dental follicle cells during in vitro differentiation with insulin and dexamethasone (dexamethasone/insulin assay). Moreover, we present atomic force microscopy (AFM) images of tissues derived from differentiated dental follicle cells.

2. Materials and methods

2.1. Cell culture

Two impacted human third molars were surgically removed and collected from two 24-year-old patients with informed consent (Fig. 1A). The mineralized teeth, including any dental hard tissues and alveolar bone fragments, were separated from the dental follicle tissue. The surfaces of the follicle tissues were cleaned and minced using a sterilized scalpel. Dental papilla tissue was discarded. Dental follicle explanted tissues were digested in a solution of collagenase/dispase (Roche, Mannheim, Germany) for 1 h at 37 °C. Minced and digested tissues were seeded out into 60 mm plates in media derived from MSCGM BulletKit media (Poietics Technologies, Gaithersburg, MD, USA) starting from the first passage. Dental follicle cells were incubated at 37 °C in 5% CO2 in a humidified atmosphere.

For dexamethasone analysis, cells were cultured as described previously (Gronthos et al., 1994). We substituted dexamethasone (10^{-8} M) with insulin (5 μg/ml final concentration) for the insulin-based differentiation protocol. Long-term cultures were made with follicle cells at passage 5 for 4 weeks with media change twice a week, but without cell passaging. After 4 weeks, cultures were washed in PBS and then fixed with 4% formaldehyde/0.1 M PBS at 4 °C for a minimum of 24 h. Calcium deposits were detected by treatment with 2% Alizarin Red S (pH 4.2) and alkaline phosphatase activity with naphtol and fast red violet (frv). Human retinal pigment epithelial cells were provided by Dr. Cinatl (University of Frankfurt, Germany).

2.2. Histology, histochemistry and immunohistochemistry

Dental follicle tissue was washed and fixed in 4% formaldehyde/0.1 M PBS at 4 °C for a minimum of 24 h. Tissues from long-term culture (4 weeks) of dental follicle cells were harvested mechanically by scraping and were fixed in 4% formaldehyde/0.1 M PBS at 4 °C for a minimum of 24 h. Both tissues were then dehydrated in an ascending series of ethanol and embedded in paraffin. Serial sections of 5 μm were cut in different planes. Haematoxylin–eosin (HE) staining was used. Selected sections were stained with trichrome, von Kossa staining and with periodic acid Schiff (PAS) staining.

For immunocytochemistry, cells were fixed with methanol/acetone and stained with vimentin antibody, diluted 1:200 (Oncogene), and Pan-Cytokeratin antibody, diluted 1:200 (CalBiochem). The specificity of the Pan-Cytokeratin antibody was checked with human retinal pigment epithelial cells. The Zymed broad-spectrum immunoperoxidase kit (Vector Laboratories) was used for detection.

Tissue sections were also subjected to immunohistochemistry. Briefly, after deparaffinization, endogenous peroxidase blocking and preincubation with TBS containing 4% bovine serum albumin, sections were incubated with the following antibodies: mouse monoclonal anti-vimentin (Oncogene, San Diego, USA), diluted 1:500 for 1 h at room temperature (rt); mouse monoclonal anti-fibronectin (Neomarkers, Westing-house, CA, USA), diluted 1:50, incubated for 1 h at rt; rabbit polyclonal anti-osteopontin (OPN) (Abcam, Cambridge, UK), diluted 1:200, incubated for 1 h at rt; and mouse monoclonal anti-bovine osteocalcin (OCN) (Takara, Otsu, Shiga, Japan), diluted 1:1000, incubated for 1 h at rt. Binding of polyclonal antibodies was detected
using the PAP method. In brief, after incubation with swine anti-rabbit immunoglobulin (DakoCytomation, Glostrup, Denmark) as secondary antibody, a PAP-complex (DakoCytomation, Glostrup, Denmark) was applied. Tissue sections were stained with DAB (Sigma Chemicals, Deisenhofen, Germany) as substratum. Monoclonal antibody binding was visualized using Envision®-Kit (DakoCytomation, Glostrup, Denmark) with DAB as substratum. Sections were counterstained with hemalaunum.

Specificity controls were included by (i) omitting the primary antibody and incubating slides with TBS or normal horse serum instead; and (ii) omitting primary antibodies or bridge and secondary antibodies. Positive controls were human tooth specimens carrying the antigens investigated.

2.3. Reverse transcription (RT)-PCR

Total RNA was isolated from cells with the RNeasy Kit (Qiagen, Hilden, Germany). Genomic DNA contamination was eliminated with RNase-free DNase in a column digestion (Qiagen, Hilden, Germany). First-strand cDNA synthesis was performed using 0.5 µg total RNA and the Omniscript Kit (Qiagen, Hilden, Germany) using an oligo-dT primer (invitrogen). The HotStarTaq Master Mix (Qiagen, Hilden, Germany) was used for conventional PCRs. For real-time PCR, the Quantitect Sybr Green Kit (Qiagen, Hilden, Germany) was used. Primers are listed in Table 1. For conventional PCRs, we used a PCRexpress thermocycler (ThermoHybaid). PCRs without reverse transcriptase were made for detection of genomic DNA. The Opticon machine (MJ Research, Waltham, MA, USA) was used for real-time PCR. The Opticon Monitor software was applied for estimation of threshold cycles. All RT-PCR products were analyzed at melting temperatures using Opticon Monitor software. For quantification, we used the delta/delta calculation method described by Winer et al. (1999) and MS Excel®. The gene expression of GAPDH was used as a reference gene in all applications.

2.4. Atomic force microscopy (AFM)

The samples were imaged with an MFP-3D atomic force microscope (Asylum Research, Santa Barbara,
CA). Cantilevers with nominal spring constants between 0.06 and 0.32 N/m and oxide-sharpened silicon nitride tips (Digital Instruments) were used in contact mode. The line scan frequency was 1 Hz. The images are unfiltered except for slope removal along each scan line to level the image.

3. Results

Dental follicle slices displayed connective tissue containing rest of dental epithelial cells (Fig. 1B), but were negative for the osteoblast marker osteocalcin (data not shown). Plastic adherent cells were isolated from two surgically removed dental follicles (dental follicle cells 1 and 2). They displayed a fibroblastoid morphology and expressed nestin and Notch-1 (Fig. 1C).

Isolated cells were positively stained for vimentin (Fig. 1 D), but were negative for Pan-Cytokeratin (Fig. 1 E), which demonstrated that only mesenchymal cells and no dental epithelial cells were obtained. We made long-term cultures with dental follicle cells at passage 5 under three different conditions with: (1) growth media containing insulin, (2) growth media with dexamethasone and (3) growth media without dexamethasone and insulin (control).

After 4 weeks of long-term culture, we harvested membrane-like structures with mineralized nodules from both differentiation approaches, which could be demonstrated with alizarin red staining and alkaline phosphatase activity (Fig. 2 A, B). However, no membrane-like structures or mineralized foci were detected in control long-term cultures. Gene expression was monitored for dental follicle cells 1 and 2 at day 28 of differentiation (Fig. 2 C). At day 28 of differentiation, OCN (dental follicle cells 1 and 2) and BMP-2 (dental follicle cells 2) gene expression was increased in both differentiation approaches. In contrast, transcripts for ameloblastin and DSPP were not detected (data not shown). Moreover, gene expression of dental follicle cells 1 was monitored during differentiation at days 0, 3, 7, 14 and 28 (Fig. 3).

Real-time RT-PCR results revealed that transcripts for nestin and BMP-2 increased during differentiation with insulin and dexamethasone and OCN starting from day 14 of differentiation. After dexamethasone and insulin treatment, culture products appeared as flat membranes consisting of a collagenous connective tissue covered by a cellular single layer (Fig. 2 D). Cells within the connective tissue had a fibroblastoid phenotype with granulated cytoplasm. We did not find any sign of lipid-containing adipocytes following induction of the follicle cells. Focal granular calcifications were dispersed over the membranes. Larger calcified areas were visible beneath the covering cell layer (Fig. 2 E). Fibroblastic cells were often arranged around globular calcified foci. Moreover, fibroblastic cells of all specimens investigated were PAS positive (Fig. 2 F). We observed the following results using immunohistochemistry:

Vimentin: In all specimens, most of the cells were reactive and showed cytoplasmic immunostaining (Fig. 4 A).

Fibronectin: In all specimens, the extracellular matrix was stained moderately or weakly. Cells of the surface appeared to be particularly immunoreactive (Fig. 4 B).

Osteocalcin: Only calcifications were stained weakly or moderately (Fig. 4 C).

Osteopontin: Moderate immunoreactivity (ir) for osteopontin could be observed in the extracellular matrix, cells and calcifications (Fig. 4 D). A representative negative control is demonstrated in Fig. 4 E.

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5’ → 3’</th>
<th>Reverse 5’ → 3’</th>
<th>Accession number or reference</th>
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<tr>
<td>Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)</td>
<td>CGTTCTCACACCACTGGAGA</td>
<td>CGGCCATCAGCAGAGCTTT</td>
<td>M33197</td>
</tr>
<tr>
<td>Collagen 1 (Col1)</td>
<td>AGGCCCTCAAGGTTCATCAGGG</td>
<td>CCAGACCATTGTTCCCCCTAA</td>
<td>Y00724</td>
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<tr>
<td>nestin</td>
<td>GCCCTGACACCCTTACGGTTTA</td>
<td>GGAGTCCTGGATTCTCTCC</td>
<td>BCO51373</td>
</tr>
<tr>
<td>Notch-1</td>
<td>GCACCTGGCAAACCTACAC</td>
<td>AGGCACCTGCGACCATC</td>
<td>AF308602</td>
</tr>
<tr>
<td>BMP-2</td>
<td>AACACTGTGCGCAGCTTCC</td>
<td>CATAGGTTAGCCTTCCT</td>
<td>Gronthos et al. (2002)</td>
</tr>
<tr>
<td>Bone sialoprotein (BSP)</td>
<td>CATATCCAGAGAGCCTTCC</td>
<td>AGAGGAGACCCCTAGAC</td>
<td>Gronthos et al. (2000)</td>
</tr>
<tr>
<td>Osteocalcin (OCN)</td>
<td>CCATAGAAACCTTTCAAGAAATATT</td>
<td>TAGAAAACCTTCCCTCCTAC</td>
<td>Buchaille et al. (1998)</td>
</tr>
<tr>
<td>Dentin sialoprophosphoprotein (DSPP)</td>
<td>GCCATAGGGCGCACTTCC</td>
<td>CAGGCGCAGACCCCTATT</td>
<td>Buchaille et al. (1998)</td>
</tr>
<tr>
<td>Ameloblastin</td>
<td>GCATAGAAGCTTACCTTCAAA</td>
<td>AATAGTGTCTCTGCTTTCTAGG</td>
<td>Gronthos et al. (2000)</td>
</tr>
<tr>
<td>runx2</td>
<td>CAGGCGCAGACCCCTATT</td>
<td>CAGGCGCAGACCCCTATT</td>
<td>Gronthos et al. (2000)</td>
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Fig. 5 shows the surface from mineralized tissue derived from differentiated dental follicle cells with insulin. The surface is mainly smooth and interrupted with blebs (Fig. 5A, arrowheads) and fibers (Fig. 5B, arrows) or rough with unorganized fiber bundles.
Fig. 2. Differentiation of dental follicle cells: (A, B) adherent layers of cultured dental follicle cells (passage 5) are shown with alkaline phosphatase activity (A) and Alizarin Red staining (B) following 4 weeks of induction with DMEM with 10% FCS, L-ascorbate-2-phosphate, inorganic phosphate and with insulin or dexamethasone and long-term culture without dexamethasone or insulin for control (orig. magnification: 40×). (C) Real-time RT-PCR for relative gene expression of dental follicle cells at passage 5 after long-term culture (4 weeks). Long-term cultures were made for dental follicle cell differentiation with growth media with insulin or dexamethasone (and without dexamethasone or insulin for control). Samples were measured in duplicate and mRNA derived from dental follicle cells at passage 5 was used for calibration (day 0 of differentiation, relative gene expression = 1). Primers were used for BSP, OCN, CollI, BMP-2, Notch-1 and nestin. (D)–(F) Paraffin slices of tissues derived from differentiated dental follicle cells treated with dexamethasone or insulin: (D) dexamethasone treatment; asterisks: single cellular layer, black arrows: focal calcifications; H.E., bar: 21 μm; (E) dexamethasone treatment, von Kossa staining; black stained areas corresponds to calcified structures, black and white arrows: superficial calcifications; bar: 21 μm; (F) insulin treatment; PAS staining; PAS-positive fibroblastic cells embedded in collagenous connective tissue matrix; bar: 21 μm.
Fig. 3. Real-time RT-PCR for relative gene expression of dental follicle cells 1 at passage 5 derived from long-term cultures (4 weeks). Long-term cultures were made for dental follicle cell differentiation with growth media with insulin or dexamethasone and for control with growth media only. Two replicates were used for each approach and mRNA derived from dental follicle cells at passage 5 was used for calibration (day 0 of differentiation, relative gene expression = 1). Primers were used for BSP, OCN, Col1, BMP-2, Notch-1 and nestin. Each bar represents the average of two experiments. Error bars denote the range between experiments.
4. Discussion

Friedenstein and colleagues (Friedenstein, 1980; Owen and Friedenstein, 1988) documented isolation of osteoprogenitor cells from aspirates of bone marrow by their ability to adhere to a plastic substratum. Recently, dental stem cells or precursor cells have been isolated by plastic adherence and clonogenic properties (Gronthos et al., 2000; Seo et al., 2004). In this study, dental follicle cells were isolated from two individual human dental follicles by their ability to adhere on a plastic surface. Both isolated cell lines were able to differentiate into a membrane-like structure with mineralized foci. We applied osteogenic differentiation protocols with dexamethasone and insulin.

The process of osteogenic differentiation can be divided into several steps, consisting of cell proliferation, extracellular matrix synthesis, maturation and mineralization. Differentiated dental follicle cells were characterized with histological and immunohistochemical methods. Fibronectin was stained moderately or weakly in all specimens as a marker for extracellular matrix maturation. The immunohistochemical detection of fibronectin in the cultured specimens may indicate its role in extracellular matrix organization and in osteogenic differentiation processes. However, fibronectin is also found in adult human PDL (Embery et al., 2000). Moreover, markers for mineralization, like OCN and OPN, were also detected in specimens treated with insulin and dexamethasone.

We investigated gene expression of OCN, BSP, BMP-2 and Coll with real-time RT-PCR technology for both differentiation protocols. The expression of OCN and BMP-2, which are markers for osteogenic differentiation in vitro (Frank et al., 2002), increased during in vitro differentiation. Zhao et al. (2002) reported that BMP-2 promotes differentiation of immortalized murine dental follicle cells towards an osteoblast or cementoblast phenotype. BMP-2 expression was also increased during osteogenic differentiation in a similar approach with BMSCs (Frank et al., 2002). OCN transcripts increased by about 110-fold at day 28 of differentiation in insulin-treated cells, compared to untreated cells at day 0 of differentiation (Fig. 2C).

Immunohistochemically, OCN could be found in calcification foci of insulin-treated specimens. Interestingly, we observed a decrease of BSP expression during differentiation. Recently, down regulation of BSP expression has been observed in a study on PDL cell differentiation from mesenchymal stem cells (Kramer et al., 2004). Interestingly, runx2 (also known as cbfa1) gene expression was not increased during differentiation.

(Fig. 5B (stars), C, D). These fibers have a periodicity of 75 nm, indicating collagen fibers (Fig. 5C).
It is known that PDL cells possess mechanisms to inhibit expression of runx2 (Kato et al., 2005). Moreover, DSPP and ameloblastin transcripts associated with (pre)odontoblasts or (pre)ameloblasts were not detected (Buchaille et al., 1998; Bégue-Kirn et al., 1998). All these data support in vitro differentiation of human dental follicle cells into a membrane-like tissue containing cells related to PDL cells.

Plastic adherent dental follicle cells expressed vimentin, a mesenchymal marker. Moreover, Notch-1 transcripts were also detected in dental follicle cells. Notch-1 is a transmembrane protein important in various cell fate decisions during development (Artavanis-Tsakonas et al., 1999). This protein has also been associated with murine dental stem cells (Harada et al., 1999). However, a detectable gene expression of Notch-1 during differentiation with both insulin and dexamethasone treated dental follicle cells could be measured. The nestin gene expression was increased during long-term cultures with dexamethasone and insulin in human dental follicle cells. During tooth development, nestin is first expressed at the bell stage. Its distribution is restricted to dental pulp cells located at the cusp area of the fetal teeth, but it is absent from the dental follicle (About et al., 2000). However, nestin is also expressed at all stages of murine tooth development, for example, in odontoblasts, both during odontogenesis and after tooth eruption (Terling et al., 1995). Further studies have to be done to evaluate the function of Notch-1 and nestin during tooth development.

The structure of the mineralized tissue was investigated by AFM. Two different structures appeared in AFM images: (1) a smooth structure interconnected by thin fibers (Fig. 5, arrows) and blebs (Fig. 5, arrowheads) and (2) a rough structure with unorganized fiber bundles (Fig. 5, stars). We think that blebs and fibers are probably mineral clusters (plates) and collagen fibers, respectively. The rough structure is comparable to published AFM images made from bone and cementum (Hassenkam et al., 2004; Ho et al., 2004). It is known that cementum structure is similar to bone (Ten Cate et al., 2003). Additionally, we found no published AFM images of dentin similar to our images (Balooch et al., 2001). Our structural data support osteogenic differentiation of dental follicle cells in this study.

Fig. 5. AFM deflection images (A–C) and AFM height image (D) of the surfaces of mineral tissue derived from differentiated dental follicle cells with insulin. Arrows: fibers; arrowheads: blebs; stars: unorganized fiber bundles.
Recently, PDL stem cells were isolated as rapidly proliferating and colony forming cells from a pooled cell population of different PDL tissues (Seo et al., 2004). Young et al. (1999) examined isolated fibroblastic cells for identification of progenitor cells and multipotent cells in a comparison/contrast analysis using dexamethasone and insulin. The presence of multipotent cells and progenitor cells was concluded from different qualities and quantities of phenotypes of differentiated cells (Young et al., 1999). It was assumed that if phenotypes are seen at the same level of insulin and dexamethasone, then the culture contains only progenitor cells. We identified similar phenotypes for insulin and dexamethasone treated cells after 4 weeks of differentiation. This work demonstrates differentiation of dental follicle cells with an insulin-based protocol for the first time. Further studies will clarify the relationship of human dental follicle cells to human PDL stem cells.

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