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Influence of Resinous Monomers on the Differentiation *in Vitro* of Human Pulp Cells into Odontoblasts

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Abstract: Odontoblasts are highly differentiated postmitotic cells, which under pathological conditions such as carious lesions and dental injuries may degenerate and be replaced by other pulp cells. A recent work showed that this physiological event can be reproduced in an *in vitro* assay system. The purpose of the present study was to evaluate the effects of resinous monomers on odontoblast differentiation *in vitro*. Pulp cores from extracted human third molars were cultured with β -glycerophosphate (2 mM) and used to evaluate the effects of TEGDMA, HEMA, UDMA, and Bis-GMA on the differentiation of pulp fibroblasts into odontoblasts. The effect of the monomers was studied by evaluating the expression of several odontoblast specific genes. In the absence of monomers, mineral nodule formation was observed. Pulp cells contributing to the nodule formation synthesized type I collagen, osteonectin, and dentin sialoprotein (DSP). In addition, Fourier transform infrared microspectroscopy showed that the mineral and organic composition of the nodules were characteristic of dentin. When the monomers were added at nontoxic concentrations, the effects of HEMA and Bis-GMA were more evident than that of TEGDMA and UDMA on collagen 1, osteonectin, and DSP expression. However, all monomers significantly decreased DSP expression and completely inhibited the mineral nodule formation.

Keywords: tooth dentin; human pulp odontoblast differentiation; culture; resinous monomers

INTRODUCTION

Dental pulp cells and dentin can be seen as an entity called pulp–dentin complex.¹ This means that factors affecting the dentin may have consequent effects on the dental pulp. This is extremely important during dental restorative procedures, because the preparation of the cavity and its subsequent filling by different materials may be harmful for the dental pulp.

During the last 40 years, many studies have shown that dental cavity preparations may cause pulp injuries because of the excessively high temperature, vibrations, and pressures generated by the bur onto the dentin surface.² The pulp reactions are variable, but it has often been shown that a deep drilling into dentin leads to the disruption of the odontoblastic layer.³ Subsequently, the restorative material, placed onto the

dentin cavity, may also produce a pulp reaction due to dentin permeability. The structure of dentin allows the diffusion of small molecules released from the material, as well as bacterial diffusion toward the pulp. It is well established that the initial pulp reaction is due to restorative materials: Uncured monomers leaching from composites can diffuse through dentin and reach the pulp within minutes after placement of a restorative material.⁴ In a second time, the bacterial leakage around the margins of a definitive restoration is the principal reason of pulp pathology observed under a restorative material.^{5,6}

The cytotoxicity of a restorative material can be measured by determining the ID50: a concentration cytotoxic to 50% of the cells. The resinous monomers are cytotoxic on pulp fibroblasts and their ID50 ranges from 10^{-5} to 10^{-2} M.⁷ The degree of cytotoxicity depends on the concentration of the material used, and the monomers may present synergistic or antagonistic cytotoxic effects.⁸ At noncytotoxic concentrations, the monomers may reduce the mitochondrial activity of pulp macrophages⁹ and suppress interleukin-1 and tumor necrosis factor secretion.¹⁰ They may also cause T lympho-

cyte immunosuppression¹¹ and complement activation,¹² thus playing an important role in pulp response. All these *in vitro* studies have shown the undeniable effects of the restorative materials on dental pulp cells. Pulp reactions could be even more pronounced when the remaining dentin is thin.¹³

Direct pulp capping involves the placement of the filling material directly onto the pulp core, which is partly amputated of its odontoblastic layer. During pulp healing, pulp cells may differentiate into odontoblast-like cells, which subsequently start to secrete the matrix of the reparative dentin.¹⁴ The mechanisms leading to odontoblast differentiation and reparative dentin secretion, during dentin wound healing, are not completely understood.^{15–17} Controversy exists concerning the safety of the direct pulp capping with resinous materials.¹⁸ The resinous monomers may play a role in the phenomena that lead to odontoblast differentiation indispensable to pulp repair. Here this hypothesis is tested in an *in vitro* culture system of human pulp cells.

MATERIALS AND METHODS

Materials

Reagents. All materials used for culture media preparation were purchased from Gibco BRL (Life Technologies Inc., Grand Island, NY) unless otherwise noted. Minimum essential medium (MEM) was supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 UI/ml penicillin, 100 μ g/ml streptomycin (Biowhittaker, Gagny, France) and 0.25 μ g/ml amphotericin B (Fungizone®).

Teeth. Normal immature third molars freshly extracted from 16–18-year-old patients were used after informed consent was obtained.

Antibodies. Polyclonal antibodies against the type I collagen were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Monoclonal antibody to osteonectin was obtained from Takara Biochemicals (Takara Shuzo Co. Ltd., Shiga, Japan). Preparation and characterization of the polyclonal antibodies against dentin sialoprotein have been already described.^{19–21}

Methods

Culture of Dental Pulp Cells. Immediately after the extraction, the teeth were swabbed with 70% (v/v) alcohol and then washed with sterile phosphate buffered saline (PBS, 0.01 M, pH 7.4). The teeth were then transferred into a laminar-flow tissue culture hood in order to perform the rest of the procedures under sterile conditions. The apical part of the teeth was removed with sterile scalpels, and the dental pulps were gently removed with forceps. Dental pulps were minced with scalpels and rinsed with PBS. The explants were cultured in 100-mm-diameter culture dishes (Becton Dickinson Labware, Lincoln Park, NJ) in MEM medium. The cultures were maintained at 37 °C in a humidified atmosphere of 5%

CO₂, 95% air. The culture medium was changed every other day. Confluent cultures were collected by trypsinization (0.2% trypsin and 0.02% EDTA) and subcultured.

Resinous Monomers Treatment. In order to evaluate the effect of resinous monomers on the differentiation of pulp fibroblasts into odontoblasts, the cultured cells were incubated in the same medium supplemented with 2 mM β -glycerophosphate (Sigma Chemical Co., St. Louis, MO). Cells incubated with β -glycerophosphate were divided into two groups: with (test group) or without (control group) resinous monomers. In the test group, the cells were incubated with one of the following resinous monomers: Tri (ethylene glycol) dimethacrylate (TEGDMA, 10 μ M), 2-hydroxyethyl methacrylate (HEMA, 10 μ M), urethane dimethacrylate (UDMA, 1 μ M) and bisphenol-glycidyl methacrylate (Bis-GMA, 1 μ M). The cells were cultured for 4 weeks and the culture medium containing the monomers was changed every other day. After culture, the cells were fixed with 70% ethanol for 1 h at 4 °C and processed for histochemistry and immunohistochemistry. The effect of the monomers on the cytodifferentiation was evaluated by studying the specific protein expression of control cells compared to that of cells cultured with the monomers.

Histochemistry for Alkaline-Phosphatase. Alkaline-phosphatase enzyme activity was revealed by incubating the cells for 30 min at 37 °C with nitro blue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate as a substrate (Sigma Chemical Co., St Louis, MO).

Immunohistochemistry. The cells were permeated for 15 min with 0.5% Triton X-100 in PBS. Primary antibodies were diluted in PBS containing 0.1% bovine serum albumin (BSA). The incubation with primary antibodies was performed overnight at 4 °C at the following concentrations: anti-collagen I antibodies were used at 40 μ g/ml, and anti-onectin at 50 μ g/ml. Antidentin sialoprotein antibody was diluted 1:200 in PBS. The immunostaining was done with the labeled streptavidin-biotin kit (LSAB; Dako Corporation, Carpinteria, CA) according to the manufacturer's instructions. Glycergel was used as a mounting medium (Dako Corporation, Carpinteria, CA). Controls were performed by incubations without primary antibodies.

Mineralization Study on Cultured Cells. The cells were plated at 3×10^3 /cm² on sterile 10-mm-diameter calcium fluoride (CaF₂) cover slips (Sorem, Pau, France) placed in four-well tissue culture plates (Nunc, Roskilde, Denmark). The cells on the cover slips were cultured with or without the monomers for 4 weeks. FTIR-MS examinations were carried out on CaF₂ cover slips fixed with absolute ethanol at 4 °C for 1 h.

Fourier-Transform Infrared Microspectroscopy (FTIR-MS). FTIR-MS examinations were recorded in the transmission mode on a FTIR (Bruker IFS55) spectrometer (Bruker,

Figure 1. Human dental pulp cells after β -glycerophosphate treatment for 4 weeks. (A) Human dental pulp cells are able to produce mineral nodules in culture. (B) Histochemistry reveals that dental pulp cells express a high level of alkaline phosphatase activity at the sites of mineral nodules formation. Original magnifications A, $\times 10$; B, $\times 10$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Karlsruhe, Germany) equipped with narrow-band mercury cadmium telluride detector responsive from 600 to 4000 cm^{-1} coupled with an infrared microscope A590 (Bruker, Karlsruhe, Germany). FTIR-MS analysis was performed at a spatial resolution of approximately 20 μm , with a spectral resolution of $\pm 2 \text{ cm}^{-1}$. The signal to background ratios were greater than 1200. Interferences from atmospheric water and CO_2 were removed by appropriate spectral subtraction techniques. Each spectrum was obtained under nitrogen purge by

the co-addition of 200 interferograms. The resulting mean interferogram was treated by the Fourier mathematical algorithm in order to obtain the corresponding absorbency spectrum.

RESULTS

Characterization of Cultured Human Dental Pulp Cells

Human dental pulp cell adhesion and proliferation were observed after 3 days of culture. After 2 weeks, the cells acquired a multilayered appearance at some areas. This was followed by the deposition of mineral crystals at these sites, which are the sites of appearance of newly formed mineral nodules. These mineral nodules were visible after β -glycerophosphate treatment for 2 weeks and continued to grow and increase in size. The cells in direct contact with the nodules exhibited a polarized morphology similar to that observed in vivo [Figure 1(a)].

The cells growing in a multilayered fashion at the sites of appearance of the mineral nodules express a high level of alkaline phosphatase activity [Figure 1(b)]. Type I collagen immunoreactivity was strong and uniform in all β -glycerophosphate treated cells [Figure 2(a)]. The expression of col-

Figure 2. Effect of UDMA (1 μM) on pulp cell cytodifferentiation and specific gene expression in the presence of β -glycerophosphate. Immunohistochemistry was used to evaluate the effect of resinous monomers on specific gene expression by pulp cells in the presence of β -glycerophosphate. In the control cultures collagen 1 (A), osteonectin (B), and dentin sialoprotein (C) are expressed in dental pulp cells and in mineral nodules. Note that expression of osteonectin and dentin sialoprotein is strong in the nodules and cells forming the nodules. UDMA (1 μM) is used here as an example to illustrate the effects of the resinous monomers on specific gene expression. When added to the culture medium for 4 weeks, UDMA slightly decreased collagen 1 (C); moderately affected osteonectin (E), but strongly decreased DSP expression (F). Mineral nodules were not observed in the presence of the monomers (D)–(F). Original magnifications $\times 10$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

lagen 1 was also evident in the mineral nodules. Osteonectin [Figure 2(b)] and dentin sialoprotein [Figure 2(c)] were expressed in these cells as well as in the nodules. However, the immunostaining intensity was higher in cells forming the mineral nodules than the cells away from these nodules. All controls where the cells were incubated without primary antibodies gave negative results (data not shown).

Effects of Monomers on the Dental Pulp Cell Differentiation

After the addition of any of the monomers, mineral nodules were not observed [Figures 2(d)–2(f)] When the monomers were added to the culture medium, they differentially affected the protein expression in dental pulp cells (Figure 3): HEMA and Bis-GMA dramatically decreased collagen 1, osteonectin and DSP immunoreactivity. UDMA [Figures 2(d)–2(f)] and TEGDMA slightly decreased collagen 1, moderately affected osteonectin, but strongly decreased DSP expression.

FTIR-MS Examinations of the Mineralization

In the control group (without monomers), the bands and peaks correlating to phosphate ions are observed (lower spectrum) at 872, 960, 1024, and 1095 cm^{-1} and for the amides at 1550 and 1640–1650 cm^{-1} . When the cultures were incubated with HEMA (upper spectrum), the bands and peaks correlating to phosphate ions were not observed (Figure 4).

DISCUSSION

Interactions between dental epithelium and mesenchyme lead to the differentiation of dental pulp mesenchymal cells into odontoblasts. These cells are postmitotic and express specific

Figure 3. Qualitative immunohistochemical evaluation of the effect of resinous monomers on pulp cell cytodifferentiation and specific gene expression in the presence of β -glycerophosphate. When the monomers were incubated with the pulp cell cultures for 4 weeks: HEMA (10 μM) and Bis-GMA (1 μM) dramatically decreased collagen 1, osteonectin, and DSP level. TEGDMA (10 μM) and UDMA (1 μM) slightly decreased collagen 1; moderately affected osteonectin, but strongly decreased DSP expression. Moreover, after the addition of any of the monomers mineral nodules were not observed. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 4. FTIR-MS of the mineralization in vitro. The lower spectrum is obtained from control cultures (without monomers) incubated with β -glycerophosphate for 4 weeks. The bands and peaks correlating to phosphate ions are observed at 872, 960, 1024, and 1095 cm^{-1} and for the amides at 1550 and 1640–1650 cm^{-1} . The spectrum is similar to the one obtained from the dentin in vivo. When the cultures were incubated with HEMA (upper spectrum), the bands and peaks correlating to phosphate ions were not observed. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

gene products that form the organic components of the dentin. Although the inorganic composition of dentin consists mainly of hydroxyapatite, collagenous and noncollagenous proteins are detected in the dentin. The main collagen found in dentin is the type I collagen (86%), whereas type III, IV, and V collagens are detected in fewer quantities. Dentin noncollagenous proteins such as osteonectin, osteocalcin, osteopontin, and bone sialoprotein have been also localized in bone. However, two extracellular matrix proteins have been shown to be tooth-specific: the dentin sialoprotein (DSP) and the dentin phosphoprotein (DPP)^{20–23} During dentinogenesis, odontoblasts secrete an unmineralized fibrillar network called predentin. The predentin layer is progressively transformed to dentin as odontoblasts transport and deposit minerals.²⁴ The mineralization of predentin involves several key regulatory events that convert the noncalcifying collagen matrix to a structure in which apatite crystals are formed and grow within and around collagen fibrils.¹⁹ The mechanisms involved in the mineralization of predentin in order to form dentin are not well understood, although several results strongly suggest that the transformation involves the secretion of noncollagenous proteins at the mineralization front.¹⁹ Thus, the deposition of these noncollagenous proteins, including the DSP, may result in both the initiation and the control of mineralization.

Previous work has demonstrated that, in spite of the absence of the tubuli in the matrix produced in vitro, the organic as well as the mineral composition of the mineral nodules has a composition similar to the dentin. This confirmed the analogy between the FTIR-MS spectra obtained in vitro and those of the dentin in vivo.¹⁷

Similarly, the extracellular matrix deposits progressively form nodules of mineralized material in the control group in the present study. Immunohistochemistry revealed that the type I collagen and osteonectin were highly expressed in

these nodules. In addition, the cells express DSP, which is a 53-kDa glycoprotein.²¹ DSP accounts for 5–8% of the dentin extracellular matrix, is localized only in dental tissues, and its expression is confined to differentiating odontoblasts, with a transient expression in the presecretory ameloblasts.²¹ Because DSP is involved in the mineralization, the absence of DSP secretion may explain the complete inhibition of mineral nodules by the resinous monomers. This protein, which is expressed during human tooth development,^{20,25–26} was also expressed in the nodules, and its expression was stronger in the nodule-forming cells. Moreover, the cultured cells particularly those involved in the mineral nodules formation express a high level of alkaline phosphatase activity indicating a high mineralization potential of these cells.

When the monomers were added to the culture medium, they differentially affected the expression of these proteins. The effects of HEMA and Bis-GMA on collagen 1, Osteonectin and DSP expression were more evident than those of TEGDMA and UDMA. However, all monomers significantly decreased DSP expression and completely inhibited the mineral nodule formation. This is an interesting aspect of the effects of resinous monomers on their target cells because the resinous monomers are cytotoxic at high concentrations, but when they were used at low concentrations, they seem to provoke specific effects on pulp cells.

Bis-GMA and HEMA showed more pronounced effects than UDMA and TEGDMA. The cytotoxicity of acrylates and methacrylates depends upon their structure and their hydrophobic characteristics.²⁷ Their effect on cell differentiation may also depend on their structure. The clinical problem comes from the ubiquitous presence of Bis-GMA and HEMA, which have shown the strongest effects on cytodifferentiation. Kitasako et al.²⁸ have already shown various results when performing direct pulp capping with resins according to the material.

Although this work shows clearly that the uncured monomers affect odontoblast differentiation, the clinical consequences of the study are difficult to evaluate. The results of this work raise questions about the direct bonding of resin onto the pulp core. It has been suggested that acidic agents and adhesive resins should be avoided in vital pulp therapy.¹⁸ It has been shown that pulp repair was not obtained even 60 days after All Bond 2 application onto dental pulp.²⁹ On the contrary Cox et al.³⁰ proposed transferring their histological findings to clinics and performing vital pulp therapy with resins. They found positive results with various resins applied onto pulp core and stated that odontoblast differentiation was possible due to a proper control of hemorrhage providing a stable interface for differentiation.³⁰ The results may also have clinical consequences when one is restoring a tooth with a composite. The evaluation of pulp health before performing a restoration with a composite only gives approximate information. Generally, pulp vitality is tested with a pulp tester. However, there is a lack of correlation between the clinical symptoms and the real pulp state.³¹

Dental restorations may be performed on teeth with a disturbed or partially destroyed odontoblastic layer. Under

these conditions, odontoblasts should differentiate to allow the pulp to recover completely. If the diffusing uncured resinous monomers interfere with this critical step permitting pulp healing, one may suppose that the dead odontoblasts will be eliminated but not replaced. This has recently been described in a histological study.⁶ However, dentin permeability associated to pulp clearance plays a great role, limiting diffusion of resinous monomers. No data are available regarding the clinical concentrations of resinous monomers, but decreasing concentrations may cancel the effects of resinous monomers on differentiation of odontoblasts, thus limiting the clinical effects to the direct pulp capping. For instance, subcytotoxic concentrations of HEMA induce changes in cell phospholipid metabolism in vitro,³² whereas a long exposure of THP-1 human monocyte macrophages to a sublethal concentration of HEMA modifies their mitochondrial activity, proliferation, and protein content.

The utilization of immortalized mouse odontoblast cell lines has been suggested to test the dental restorative materials because they were more sensitive than other cell lines such as the L929 fibroblast cell line.^{34,35} Although the cell lines used could be considered as target cells, the respiratory function studied cannot be considered as a specific function of these cells but rather a vital metabolic function for all eukaryotic cells.

In this work, the cells used are under the influence of dental restorative material, and the functions studied are specific to these cells. Moreover, this work highlights the effects of uncured resinous monomers and reveals that, even at noncytotoxic concentrations, the monomers affect the differentiation of odontoblasts from pulp fibroblasts and consequently the process of mineralization. Taken together, these results show that the resin monomers differentially affect odontoblast differentiation and the process of reparative dentin synthesis in vitro. This could have an important impact for clinical applications, particularly in restorative dentistry after direct pulp capping medications.

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