Cell Tissue Res (2006) 324: 225–236
DOI 10.1007/s00441-005-0117-9

REGULAR ARTICLE

George T.-J. Huang · Wataru Sonoyama · James Chen · Sang Hyuk Park

In vitro characterization of human dental pulp cells: various isolation methods and culturing environments

Received: 8 May 2005 / Accepted: 4 November 2005 / Published online: 27 January 2006

Abstract Our purpose was to characterize human dental pulp cells isolated by various methods and to examine the behavior of cells grown under various conditions for the purpose of pulp/dentin tissue engineering and regeneration. We compared the growth of human pulp cells isolated by either enzyme digestion or the outgrowth method. Expression of dentin sialophosphoprotein, Cbfa1, and two types of collagen (I and III) in these cells was examined by Western blot or reverse transcription/polymerase chain reaction. Growth of pulp cells on dentin and in collagen gel was also characterized. We found that different isolation methods give rise to different populations or lineages of pulp cells during in vitro passage based on their collagen gene expression patterns. Cells isolated by enzymedigestion had a higher proliferation rate than those isolated by outgrowth. Pulp cells did not proliferate or grew minimally on chemically and mechanically treated dentin surface and appeared to establish an odontoblast-like morphology with a cytoplasmic process extending into a dentinal tubule as revealed by scanning electron microscopy. The contraction of the collagen matrix caused by pulp cells was dramatic: down to 34% on day 14. Our data indicate that (1) the choice of the pulp cell isolation method may affect the distribution of the obtained cell populations, (2) a treated dentin surface might still promote odontoblast differentiation, and (3) a collagen matrix may not be a suitable scaffold for pulp tissue regeneration because of the marked contraction caused by pulp cells in the matrix. The present study thus provides important information and a basis for further investigations pre-requisite to establishing pulp tissue engineering/regeneration protocols.

Keywords Dental pulp cells · Dentin sialophosphoprotein · Cbfa1 · Collagen type I/III · Reverse transcription/polymerase chain reaction · Scanning electron microscopy · Human

Most of this work was performed at UCLA School of Dentistry
This study was supported in part by an Endodontic Research Grant from the American Association of Endodontists Foundation (G.T.-J.H.)

G. T.-J. Huang · J. Chen · S. H. Park
Division of Associated Clinical Specialties,
Section of Endodontics, UCLA School of Dentistry,
Los Angeles, CA, USA

G. T.-J. Huang
Division of Oral Biology and Medicine,
and Orofacial Pain, UCLA School of Dentistry,
Los Angeles, CA, USA

G. T.-J. Huang
Dental and Craniofacial Research Institute,
UCLA School of Dentistry,
Los Angeles, CA, USA

W. Sonoyama
Department of Oral and Maxillofacial Rehabilitation,
Okayama University Graduate School of Medicine,
Dentistry, and Pharmaceutical Sciences,
Okayama, Japan

G. T.-J. Huang (✉)
College of Dental Surgery, Dental School,
Department of Endodontics, Prosthodontics,
and Operative Dentistry, University of Maryland,
666 West Baltimore Street,
Baltimore, MD 21201, USA
e-mail: GHuang@umaryland.edu
Tel.: +1-410-7067285
Fax: +1-410-7063028

J. Chen
3233 Grand Ave, Ste N299,
Chino Hill, CA 91709, USA

S. H. Park
College of Dentistry,
Department of Conservative Dentistry,
Kyung Hee University,
1 Hoegi-dong, Dongdaemun-gu,
Seoul, 130-701, South Korea

Introduction

The ultimate goal of this study is to regenerate dental pulp tissue, a task that is generally considered to be difficult. Recent attempts to engineer dental pulp in vitro and in animal

models by using modern tissue engineering technologies have shed light on pulp regeneration (Bohl et al. 1998; Mooney et al. 1996). Regenerated pulp tissue should be functionally competent, e.g., capable of forming dentin to repair lost structure. Reports have shown that isolated pulp cells can be induced to differentiate into odontoblast-like cells and generate dentin-like mineral structure in vitro (About et al. 2000; Tsukamoto et al. 1992). The in vivo evidence for pulp cells being capable of generating dentin was demonstrated by Gronthos et al. (2000) who transplanted pulp cells mixed with hydroxyapatite/tricalcium phosphate into immunocompromised mice; within 6 weeks, pulp-like tissues surrounded by a dentin-like structure were observed adjacent to an odontoblast-like layer. These pulp cells isolated from adult human dental pulps and expanded in vitro were designated by Gronthos et al. (2000) as dental pulp stem cells (DPSCs). When these DPSCs are seeded onto human dentin surface and implanted into immunocompromised mice, reparative dentin-like structure is deposited on the dentin surface (Batouli et al. 2003). The studies mentioned above signify the possibility that a protocol for pulp tissue regeneration and new dentin formation for clinical therapeutic purposes can be established.

To engineer tissues in vitro, the first step is to isolate cells with the right phenotype and propagate them in suitable culturing environments. Normally, these cells are grown on a two-dimensional surface before being transferred to a three-dimensional scaffold construct (Lee and Mooney 2001; Vacanti et al. 1991; Vacanti 2003). Synthetic matrices fabricated from naturally derived (e.g., collagen) or synthetic materials (e.g., polyglycolic acid; PGA) are often utilized as a delivery vehicle for these cells and to guide the process of tissue formation (Alsberg et al. 2001; Drury and Mooney 2003; Lee and Mooney 2001). Fibroblasts cultured from human adult dental pulps seeded onto PGA form new tissue similar to that of native pulp after being implanted into mouse subcutaneous space (Buurma et al. 1999). Based on current tissue engineering principles, several questions need to be asked before establishing an in vitro protocol for pulp tissue engineering. (1) Do different pulp cell isolation methods give rise to a same pool of pulp cell population? Pulp cells have been isolated either by the well-known outgrowth method, i.e., cells outgrow from pulp tissue explants (About et al. 2000; Couble et al. 2000; Nakao et al. 2004; Saito et al. 2004; Tsukamoto et al. 1992) or by enzyme digestion (collagenase/dispase/trypsin; Gronthos et al. 2000; Nakashima 1991; Onishi et al. 1999). However, no report has compared the cells derived from the two isolation methods. (2) Will pulp cells grow on treated dentin surface? To regenerate pulp tissues in root canals that have been mechanically filed and chemically cleansed, pulp cells must be able to grow on this treated surface. (3) Is a collagen matrix a suitable scaffold? Collagen gel is a convenient carrier of pulp cells and can be injected into the canal space to regenerate pulp clinically (Lee and Mooney 2001). Unfortunately, collagen is known to contract under the influence of fibroblasts (Haas et al. 2001; Zhu et al. 2001). Would pulp cells cause the contraction of collagen to such an extent that it affects pulp tissue regeneration?

In this study, we have addressed the above questions as a first step toward establishing pulp tissue engineering/regeneration protocols. We have examined the expression of several genes related to odontoblast lineages in pulp cells isolated by the two above-mentioned methods and have characterized the growth behavior of pulp cells on a dentin surface and in a collagen matrix and compared this with growth on a culture dish. Our data presented herein provide new insights and raise new questions regarding the future directions of pulp tissue engineering/regeneration.

Materials and methods

Sample collection and cell culture

Tooth sample collection (n=24) and the growth of pulp cells followed protocols described previously (Gronthos et al. 2000; Park et al. 2004). Extraction of third molars from healthy patients (aged 16–26 years) in the Department of Oral Surgery conformed with a protocol approved by the UCLA Medical Institutional Review Board. Pulp cell cultures were established via two approaches. (1) Enzyme digestion was carried out according to Gronthos et al. (2000). Minced pulp tissues were digested in a solution of 3 mg/ml collagenase type I and 4 mg/ml dispase (Sigma, St. Louis, Mo., USA) for 30–60 min at 37°C. Cell suspensions were obtained by passing the digested tissues through a 70–μm cell strainer (Becton/Dickinson, Franklin Lakes, N.J., USA). Single cell suspensions (1×10^5 cells/flask) were seeded in 5×10 cm culture flasks containing α-minimum essential medium (α-MEM; Life Technologies/GIBCO BRL, Gaithersburg, Md., USA) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μM L-ascorbic acid-2-phosphate, 100 U/ml penicillin-G, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone (Gemini Bio-Products, Woodland, Calif., USA) and maintained under 5% CO_2 at 37°C. Cells were grown to confluence and continuously passed at a 1:3 ratio when confluent. These pulp cells were designated as human dental pulp cells/digestion method (HDPCs-d). (2) The outgrowth method was performed according to Park et al. (2004). Pulp tissue explants (2×2×1 mm fragments) were placed in 6-well plates with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics as above. The outgrown cells at confluence were transferred to 5×10 cm culture flasks (passage 1), grown to confluence, and continuously passed at a 1:3 ratio when confluent. These cells were designated as human dental pulp cells/outgrown method (HDPCs-o). The above cells obtained were harvested and kept frozen in liquid N_2 until experimentation. NIH 3T3 cells (ATCC CRL-1658) were grown in DMEM (Life Technologies/GIBCO BRL), supplemented with 10% FBS and antibiotics as above.

Cell proliferation on culture dishes

The proliferation rate of subconfluent cultures (passage 3) of HDPCs-d and HDPCs-o seeded in chamber slides (NUNC, Naperville, Ill., USA) was assessed by bromodeoxyuridine (BrdU) incorporation for 6 h, by using a Zymed BrdU staining Kit (Zymed Laboratories, San Francisco, Calif., USA). Cells were fixed and stained according to the manufacturer's instructions. Positively stained cells were counted in randomly selected areas under a light microscope. Percentages of positive cells were calculated and compared statistically by using Student's *t*-test.

Western blot analysis

Pulp cells were lysed in a buffer system (Mammalian Protein Extraction Reagent, Pierce Biotechnology, Rockford, Ill., USA), sonicated and centrifuged, and the supernatant was collected. NuPAGE LDS sample buffer (Invitrogen, Carlsbad, Calif., USA) and 2-mercaptoethanol (final concentration: 2.5%) was added to the supernatant and incubated at 70°C for 10 min. After quantitation of the collected total proteins, 5 μg protein from each sample was separated on a 10% NuPAGE gel (Invitrogen), transferred onto polyvinylidene fluoride membranes and blocked with 4% bovine serum albumin and 1% non-fat dry milk for 1 h at room temperature. The membranes were then incubated at 4°C overnight with the following antibodies against specific human proteins: mouse anti-dentin sialoprotein (DSP; LF-21, 1:500 dilution; Dr. Larry Fisher, NIDCR/NIH, Bethesda, Md., USA), rabbit anti-Cbfa1 (final concentration, 0.2 μg/ml; EMD Biosciences, Darmstadt, Germany) and rabbit anti-HSP90 (heat shock protein; final concentration, 1 μg/ml; Santa Cruz Biotechnology, Santa Cruz, USA). HSP90 was used as a loading control. The membranes were then washed and incubated with goat anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology) for 1 h at room temperature, followed by being washed and reacted with Super Signal Chemiluminescent Substrate (Pierce Biotechnology) according to the manufacturer's recommendations. Signals were visualized with Kodak BIOMAX MR films (Kodak, Rochester, N.Y., USA). The intensities of the signals were analyzed by NIH Image software ImageJ.

RNA isolation and reverse transcription/polymerase chain reaction

Total RNA of pulp cells was isolated by TRIzol (Invitrogen/Life Technologies, Carlsbad, Calif., USA). Reverse transcription/polymerase chain reaction (RT-PCR) was carried out with the RT-for-PCR kit (Clontech laboratories, Palo Alto, Calif., USA) to synthesize cDNA from 1 μg total RNA. Appropriate amounts of the cDNA, specific primers for human dentin sialophosphoprotein (DSPP), collagen I, and collagen III or for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, from RT-for-PCR kit, Clontech) and *pfu* DNA polymerase (Stratagene, La Jolla, Calif., USA) were used for PCRs as follows: 1 min at 94°C (denaturing), 45 s at 55°C (annealing), and 1 min at 72°C (extension) for 30 cycles; 7 min at 72°C as the final step. PCR products were size-fractionated on a 1% agarose gel and visualized by ethidium-bromide staining. The intensities of the PCR products were analyzed by Scion Image software (Scion, Frederick, Md., USA). The primer sequences were as follows: DSPP (target size, 488 bp), sense, 5'-GCAGTGATGAATCTAATGGC-3'; antisense, 5'-CTGATTTGCTGCTGTCTGAC-3'; collagen-I (target size, 183 bp), sense, 5'-TCAGAGAGGAGAGAGAGGCT-3'; antisense, 5'-ATTCAGGGGAACCTTCGGCA-3'; collagen-III (target size, 410 bp), sense, 5'-GCCAATCCTTTGAATGTTCC-3', antisense, 5'-CAATAGGTAGTCTCACAGCC-3'; hGAPDH (target size, 1041 bp), sense, 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3', antisense, 5'-CATGTGGGCCATGAGGCTCACCAC-3'. The DSPP gene encodes two major tooth matrix proteins dentin sialoprotein (DSP) and dentin phosphoprotein (DPP). DSP and DSPP are considered to arise from a single transcript, the product of which is cleaved into the two proteins (MacDougall et al. 1997).

Cell growth on dentin surface

Dentin specimens were prepared from collected human third molars after the pulp tissue had been removed to obtain pulp cells. The coronal dentin was cut into a disc shape (approximately 1 mm thick) with a diameter approximating that of a well of a 96-well plate, and the surface of the dentin disc was polished with 600-silicon-carbide sandpaper. The dentin discs were treated with: 17% EDTA for 10 min and 19% citric acid for 1 min to remove the smear layer (Di Lenarda et al. 2000; Froes et al. 2000); betadine and 5.25% NaOCl for 30 min each to sterilize; 1×phosphate-buffered saline (PBS; several rinses and a soak) for 2–7 days to remove residual sterilization agents. The above chemical treatments of dentin produced a replica of the dentin surface of a root canal wall subjected to cleansing during clinical endodontic procedures. HDPCs (10^4 cells) and NIH 3T3 (4,000 cells) were seeded onto each dentin disc inserted into a 96-well plate or directly onto the wells (4,000 cells/well of both HDPCs and NIH 3T3) of the 96 well-plate. At various time points, cells were trypsinized and counted to plot the growth curves.

Scanning electron-microscopic analysis of pulp cells grown on dentin discs

Pulp cells were seeded on dentin disc surfaces in 96-well with 5×10^4 cells/dentin disc. For scanning electron-microscopic (SEM) analysis, cells were washed three times with PBS, fixed in Karnovsky fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 0.02% $CaCl_2$), rinsed in cacodylate buffer, and

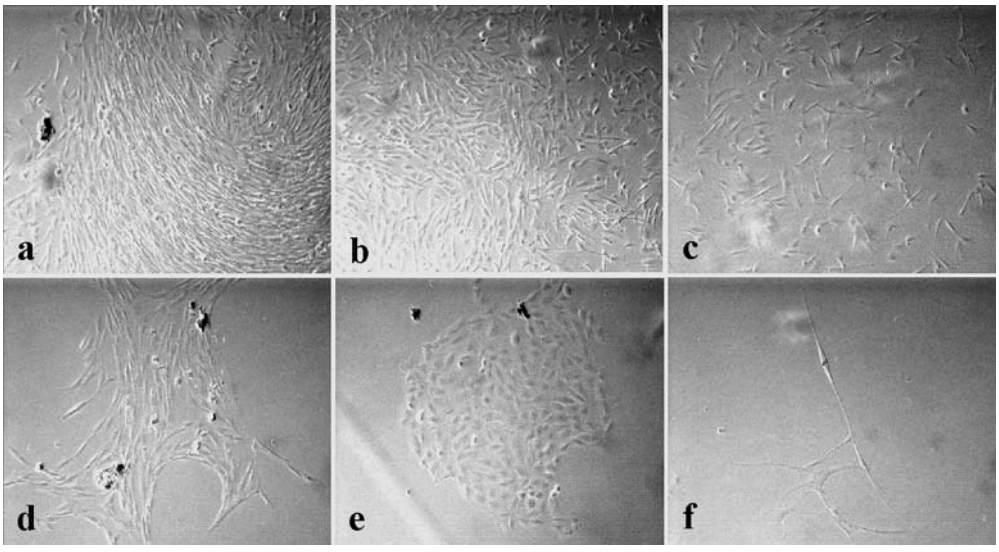

Fig. 1 HDPCs-d colonies formed after digestion and being plated in culture dishes. Colonies generally formed in 7–14 days and had the following appearance: high density (**a**), median density (**b**), low density (**c**), pattern-forming (**d**), endothelial-/epithelial-like (**e**), long slender single cells (**f**). Original magnifications: ×40

dehydrated in a graded series of ethanol. They were further dehydrated in hexamethyldisilazane and air-dried. After gold coating, the preparations were observed with a Cambridge 360 Scanning Electron Microscope (Carl Zeiss SMT, Thornwood, N.Y., USA).

Cell proliferation in collagen gel matrix

Cultured cells were trypsinized and mixed with NaOH-neutralized type I collagen solution, plated into culture wells, and incubated at 37°C, under 5% CO_2, for 60 min to allow gelation. Cell culture medium was then added to the wells, and cells in the collagen gel were removed at different time points for cell counting by incubating the gels with a 3 mg/ml solution of type I collagenase (Sigma) in PBS for 45 min.

Two types of collagen solutions were used and prepared according to maufacturer's instruction with minor modifications. (1) Rat tail type I collagen solution (3.97 mg/ml; BD Biosciences, Bedford, Mass., USA) was prepared as follows: 9 parts type I collagen, 0.023 part 1 N NaOH, 1 part 10× buffered cell culture medium (DMEM) or 10× PBS. (2) Bovine type I collagen gel solution (Vitrogen 100, 3.1 mg/ml; Cohesion, Palo Alto, Calif., USA) was prepared as follows: 8 parts type I collagen, 1 part 0.1 N NaOH, 1 part 10× DMEM. Our preliminary experiments showed no observable difference in cell growth or contraction behavior between the two types of collagen matrix. Therefore, both types were used in the cell proliferation and gel contraction studies described below.

Collagen gel contraction assays

The mixtures of cultured cells and collagen gel were placed into wells of 96-well plates (100 μl/well) and allowed gelation. Culture medium (200 μl/well) was then added into each well causing the cell/collagen mixture to lift off from the well bottom and to be suspended in the medium. The plates were incubated at 37°C under 5% CO_2, and the medium was changed every 2–3 days. The diameters of the gels were measured at various time points by using the built-in grid in an ocular lens of the microscope. Because the gel did not contract uniformly to produce a perfectly round shape, three diameters that divided the circle into six equal sections per gel were measured, and the average of the three measurements was recorded.

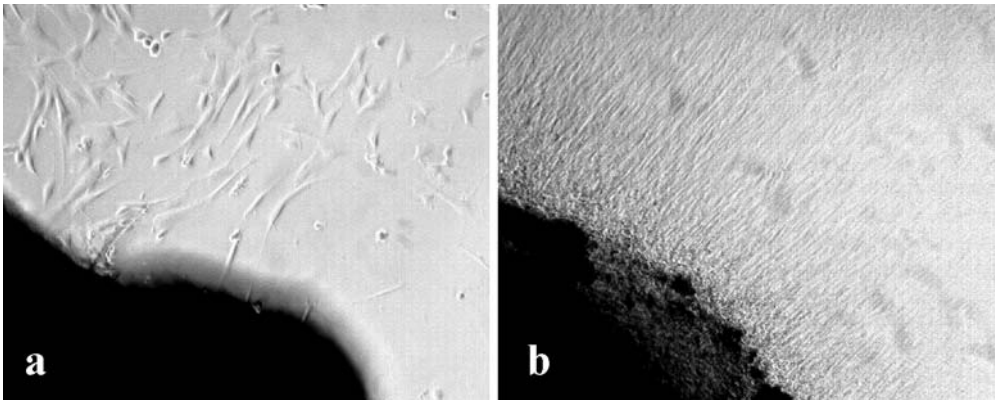

Fig. 2 HDPCs-o out-growing from a pulp fragment (*lower left dark region*). HDPCs-o at 1–2 weeks (**a**). Cells becoming confluent around the fragment in 2–3 weeks (**b**). Some pulp samples took longer to produce outgrown cells. Original magnifications: ×40

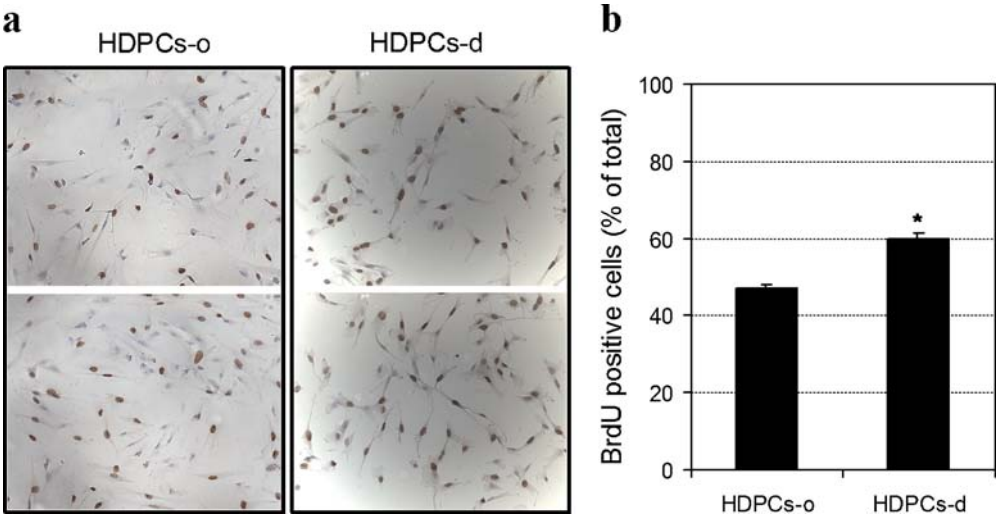

Fig. 3 Proliferation studies of pulp cells on culture dishes as measured by BrdU labeling. Pulp tissues were obtained from four third molars of one patient (aged ~20 years). Pulp cells were isolated by using the two methods described except the medium for both type of cells was the α-MEM plus the supplements, and were subcultured into chamber slides (passage 3, 2×10^4 cells/well). **a** BrdU-positive cells display brown nuclei (representative data). Original magnifications: ×200. **b** Percentage of BrdU-positive cells of total cells counted per selected area (n=4). *P=0.0004, statistically significant (t test). *Error bars* indicate standard error (SE) of the mean

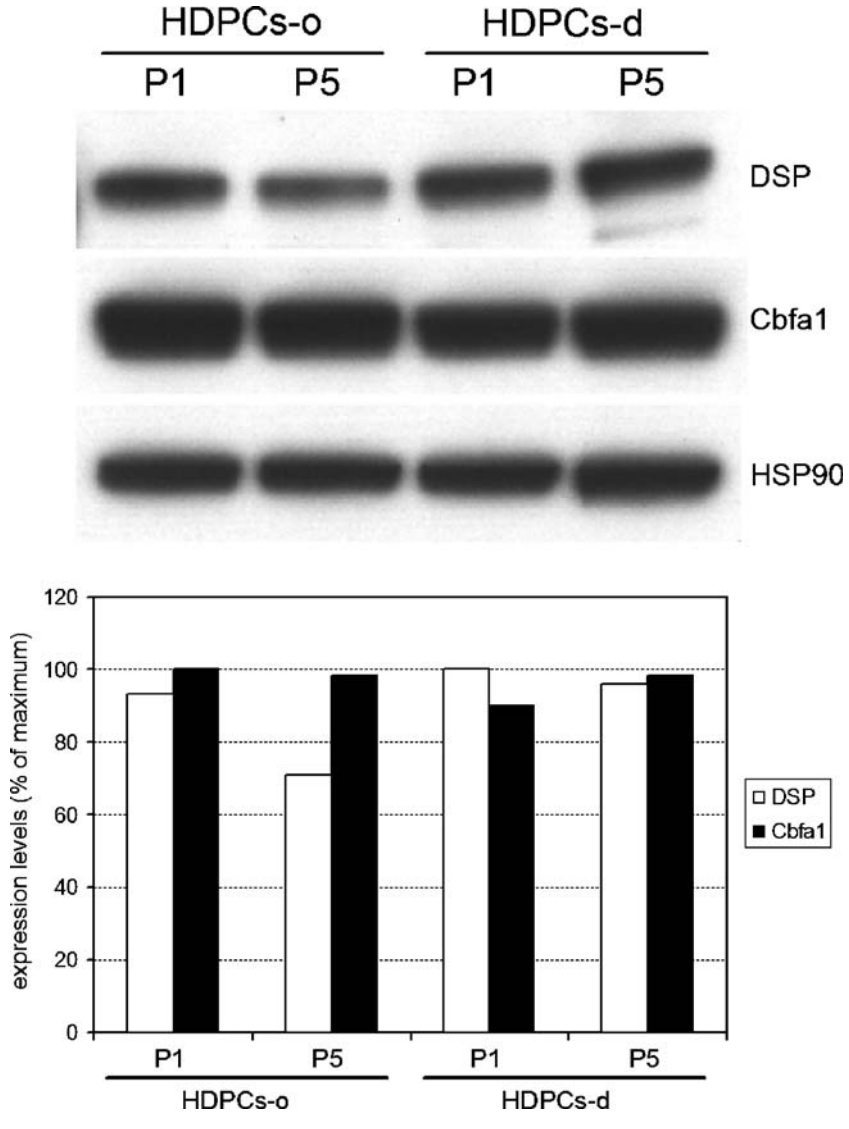

Fig. 4 *Top* Western blot analysis of DSPP (*DSP*) and Cbfa1. Pulp cells used were the same as those described in Fig. 3. At passages 1 and 5, cells were harvested, and 5 μg protein from each sample were analyzed (*P1*, *P5* cell passages 1 and 5). *Bottom* Bar chart of the relative signal intensities. Signals from each band were normalized to the corresponding HSP90 level

Results

Cell growth on culture dishes

We first observed morphological differences of pulp cells isolated by either the digestion method to obtain HDPCs-d or the outgrowth method to obtain HDPCs-o. HDPCs-d formed colonies within 1–2 weeks after initial plating, and typical colonies contained fibroblast-like cells triangular or stellate in shape (Fig. 1a–c); this was maintained after five or more passages. The appearance of the colonies of HDPCs-d in the initial stage could be categorized into three main types: (1) high to median densities (Fig. 1a,b); (2) low density (Fig. 1c); (3) spindle-shape pattern-forming cells (Fig. 1d). Other different types of colonies (minority) included cells of endothelial-like or epithelial-like morphology (Fig. 1e), and single cells with long extending processes (Fig. 1f) were also observed. HDPCs-o appeared as fibroblast-like cells with various sizes and shapes (Fig. 2a) when first migrating out of the pulp fragments. Morphological differences among these cells were more difficult to discern than in those cells that were isolated by the digestion method and that formed colonies. The HDPCs-o later became confluent and presumably represented a heterogeneous population of cells (Fig. 2b).

Proliferation on culture dishes

While characterizing the morphologies of these cells from different donors, we observed that HDPCs-d appeared to grow faster than HDPCs-o. To assess this phenomenon more accurately, we collected four third molars from one patient and divided pulp tissues in half, each undergoing the different isolation methods. At passage 3, both HDPCs-d and HDPCs-o were subjected to proliferation studies by using BrdU incorporation and Zymed BrdU staining. As shown in Fig. 3, HDPCs-d proliferated at a faster rate than HDPCs-o grown on culture dishes (Student's *t* test, $P<0.05$).

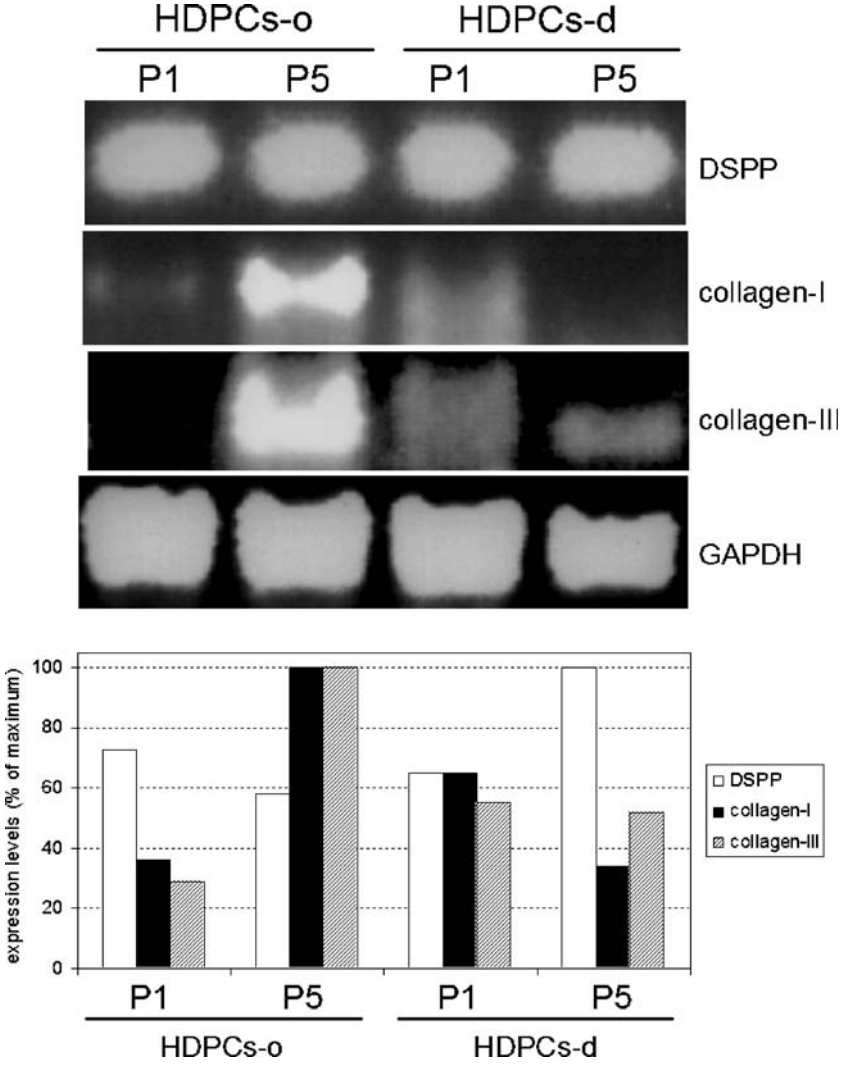

Fig. 5 *Top* Expression of DSPP and collagen types I and III in pulp cells analyzed by RT-PCR. Pulp tissues were obtained from four third molars of a 17-year-old male patient. Pulp cells were isolated with the two methods described (*P1*, *P5* cell passages 1 and 5). *Bottom* Bar chart of the relative signal intensities. Signals from each band were normalized with the corresponding GAPDH level

Expression of DSPP, Cbfa1 and collagen types I and III in cultured pulp cells

We next determined potential differences in the expression of DSPP, Cbfa1 and collagen types I and III between HDPCs-d and HDPCs-o. The data presented in Fig. 4 demonstrate protein expression of DSPP and Cbfa1 from pulp cells derived from the same donor used for the proliferation studies shown in Fig. 3. No difference were observed in the expression of the two gene products between HDPCs-d and HDPCs-o at different passages, except a slightly lower expression level of DSPP at passages 5 compared with that at passage 1 of HDPCs-o.

Pulp cells from another donor were also processed in the same manner as the above, and at different passages, RT-PCR was performed to examine the expression of DSPP and collagen types I and III. The results (see Fig. 5) showed that the expression levels of DSPP in both HDPCs-d and HDPCs-o, based on the observed signal bands, were similar at passages 1 and 5; however, after adjusting the signal intensities against GAPDH, differences were found in the expression levels between passages. DSPP levels decreased in HDPCs-o, whereas they increased in HDPCs-d from passages 1 to 5.

The expression of collagen type I changed more noticeably over passages. At passage 1, HDPCs-d expressed a higher level of type I collagen than HDPCs-o. At passage 5, the relative expression levels reversed. Collagen type I expression level in HDPCs-d decreased, whereas it increased dramatically in HDPCs-o. The relationship of collagen type III expression levels between the two types of cells was similar to that of collagen type I. At passage 1, there was minimal level of type III collagen in HDPCs-o, but this markedly increased at passage 5. For HDPCs-d, the type III collagen expression level was slightly reduced from passage 1 to passage 5.

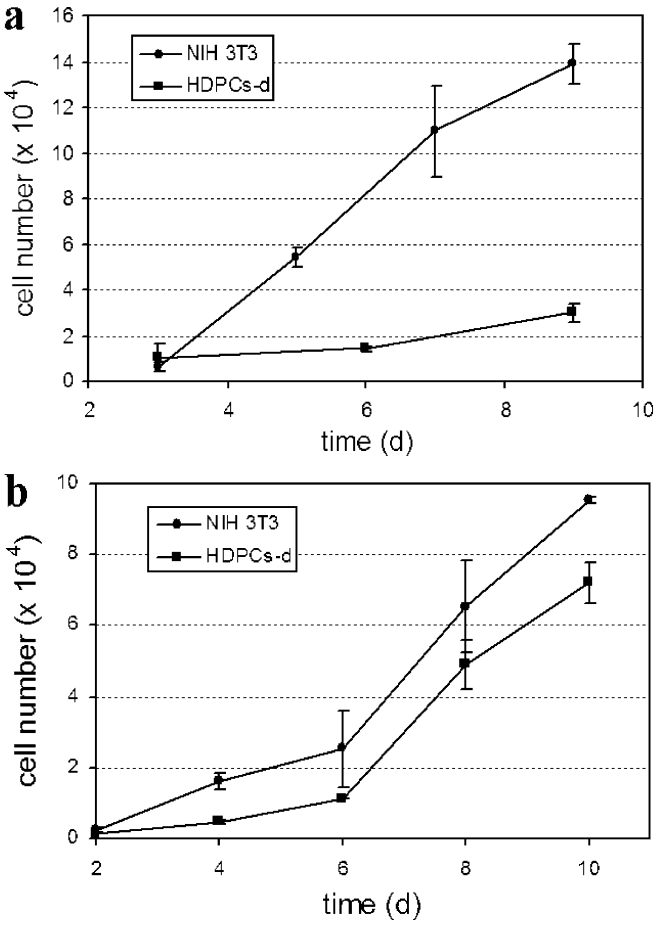

Fig. 6 Proliferation of cells grown on dentin discs or in 96-well plates. Representative experiments of cells seeded onto dentin disc surfaces (4,000 NIH 3T3 cells/dentin disc and 10^4 HDPCs-d/dentin disc) in 96-well plates (**a**) or in wells (4,000 cells/well, both NIH 3T3 and pulp cells) of 96-well plates (**b**) in duplicate or triplicate. Cells were harvested with trypsin and counted at various days after seeding. Cells were confluent on day 8 in 96-well plates (*error bars* SE). HDPCs-d were at passage 3

Cell growth on the dentin surface

According to previous reports, pulp-derived cells seeded on a dentin surface are useful for dentin barrier tests (Schmalz et al. 1996; Schmalz et al. 2001). SV40 large T-antigen-transfected bovine pulp-derived cells proliferate extensively on a dentin surface, but not to the same degree as those grown on culture dishes (Schmalz et al. 2001). To utilize isolated primary pulp cells to generate new dentin on existing dentin, it is necessary to know whether they proliferate and reach confluence on a dentin surface.

Our results showed that HDPCs-d plated onto the dentin surface proliferated minimally, if at all, compared with platings in culture dishes (96-well plates), whereas NIH 3T3 cells, which served as a comparison, proliferated similarly on dentin surfaces and in culture wells (Fig. 6). Statistical analysis revealed that the numbers of HDPCs-d on dentin discs on days 6 or 9 were not significantly different from those on day 3 (*t* test, $P>0.05$), whereas NIH 3T3 cell numbers on days 5, 7, and 9 were all significantly higher than those on day 3 ($P<0.05$). Both HDPCs-d and NIH 3T3 grown on culture dishes, in comparison, increased in cell number significantly after day 2, except for NIH 3T3 on day 6. Moreover, only 10%-20% of HDPCs-d seeded attached to the dentin surface vs ~50% of NIH 3T3 cells. HDPCs-o from a different donor were also seeded onto dentin surfaces, and similarly, no proliferation was observed (data not shown).

To visualize the cells on a dentin surface, we selected HDPCs-d and applied SEM analysis. The results (Fig. 7) agreed with the previous findings (Fig. 6), viz., HDPCs-d hardly proliferated on dentin surfaces. Cell densities appeared decreased in this representative experiment between the day-1 group (Fig. 7a) vs the day-16 group (Fig. 7b). There was also a morphological change in day-16 pulp cells on dentin discs. Instead of having a typical fibroblast-like cell morphology, as seen in the day-1 group, individual HDPCs-d appeared condensed and spherically shaped. At higher magnification, some cells were observed to possess a single cytoplasmic process extending into a

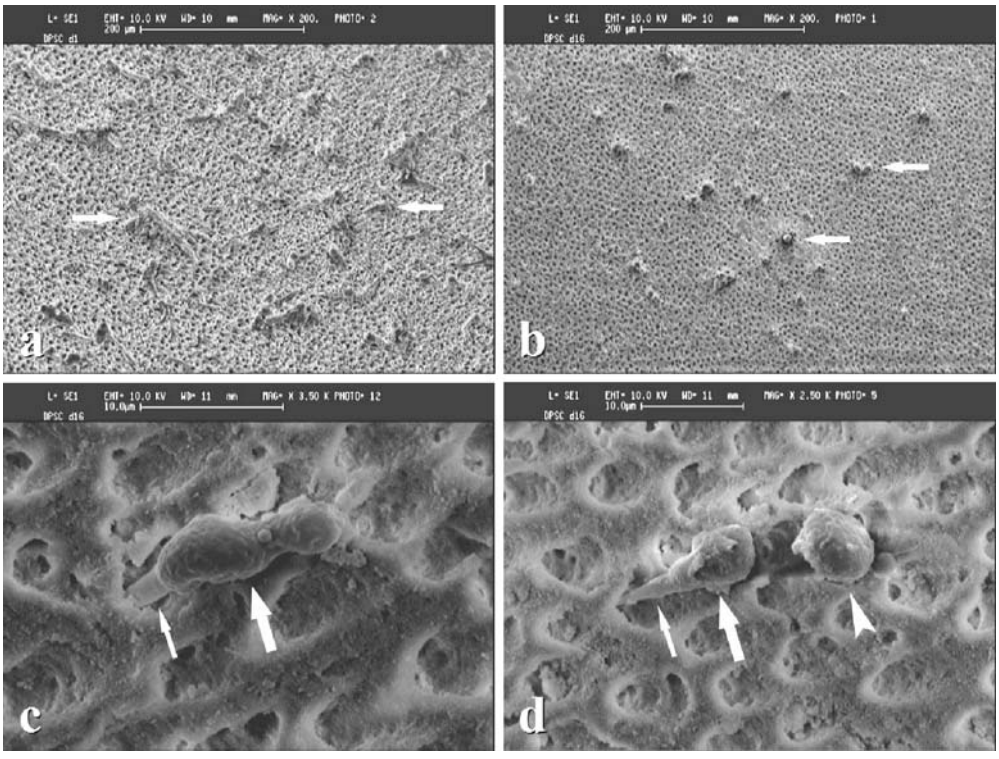

Fig. 7 SEM images of HDPCs-d (passage 3) grown on a human dentin surface. Cells were seeded onto dentin discs placed in a 96-well plate (5×10^4 cells/dentin disc) and grown for 1 day (**a**) or 16 days (**b–d**) before being fixed and processed for SEM. Medium was changed every 3 days for the 16-day group. Note HDPCs on dentin surface (*arrows* in **a**, **b**) and cell bodies of odontoblast-like HDPCs (*large arrows* in **c**, **d**), each with a process extending into the opening of a dentinal tubule (*small arrows* in **c**, **d**). One cell body, presumably of an odontoblast-like HDPC has no clearly revealed cell process (*arrowhead* in **d**), possibly because of the angle of view. Original magnifications: ×200 (**a**, **b**), ×3,500 (**c**), ×2,500 (**d**)

dentinal tubule (Fig. 7c,d) exhibiting odontoblastic characteristics, whereas such features were not observed on day 1. In particular, the polarized odontoblast-like cells had a cell body shape varying from round, to cuboidal (Fig. 7d), to columnar (Fig. 7c), a common presentation of odontoblasts at different stage of maturity (D'Souza 2002; Smith 2002).

Cell proliferation in collagen gel matrix

Collagen is a commonly used scaffold matrix to grow cells in three-dimensions, especially for the purpose of tissue engineering. Therefore, the determination of the growth of pulp cells in collagen matrix is important in order to know the suitability of collagen as a matrix for pulp tissue engineering.

HDPCs-d and HDPCs-o (from different donors) were both cast in collagen gel, and NIH 3T3 was used as a comparison. Pulp cells and NIH 3T3 proliferated in collagen gels (pH 7.0), but much more slowly than in culture dishes (Fig. 8). On days 11 and 14, all three types of cells increased in cell number significantly compared with those on day 1 or day 2 ($P<0.05$). In several independent experiments, we noted that both HDPCs-d and HDPCs-o caused contraction of the gel constructs at some points after casting. The reduced gel volume after contraction limited the cell growth. In the experiment presented in Fig. 8, HDPCs-d had a much slower growth than HDPCs-o after day 11, because HDPCs-d started to show gel contraction

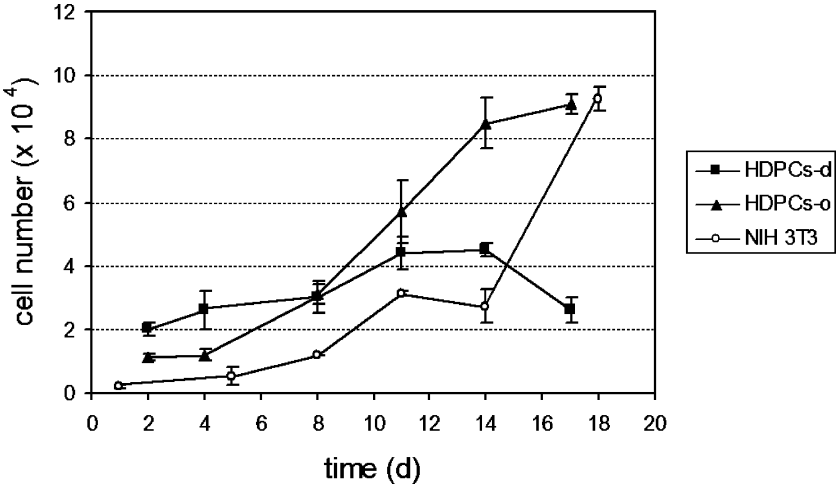

Fig. 8 Proliferation of cells grown in collagen gel. Representative experiment (performed in duplicate or triplicate) of cells mixed in a collagen gel solution and placed into 96-well plates ($0.2–2\times10^4$ cells in 0.1 ml/well). After gelation, 0.2 ml culture medium was added into each well and changed every 2 days. At each time point, cells were removed for cell counting by incubating the gels with type I collagenase (*error bars* SE). Rat tail collagen (pH 7.0) was used for NIH 3T3 and bovine collagen (pH 7.0) for HDPCs-d (passage 2) and HDPC-o (passage 4)

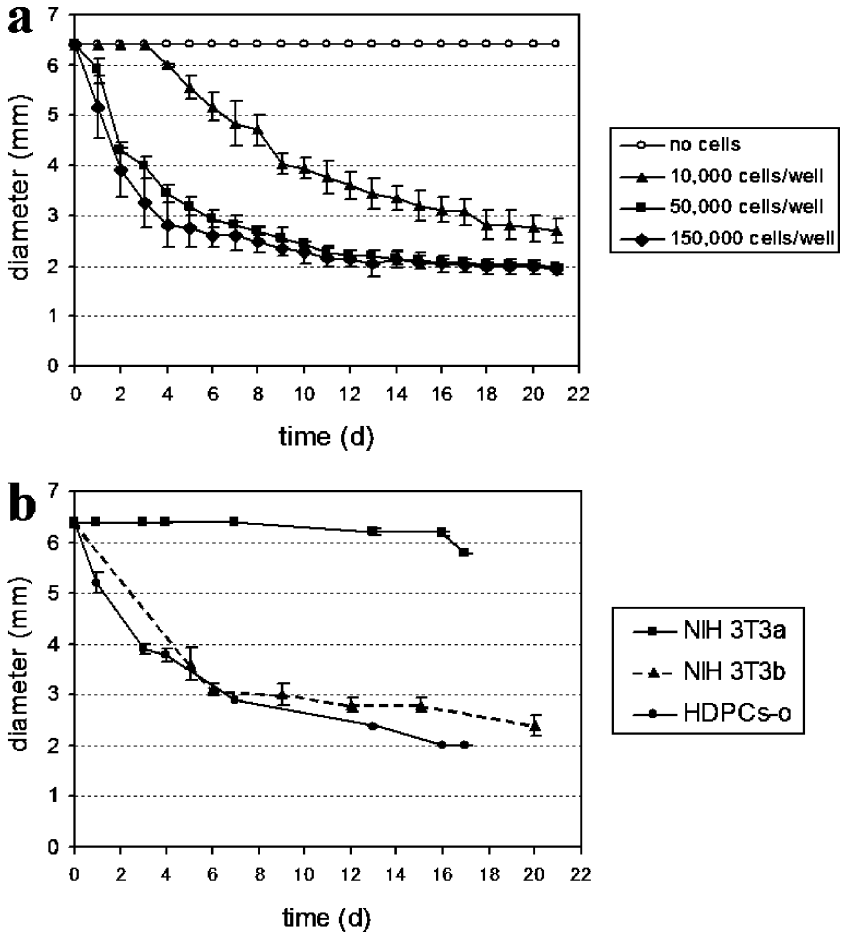

Fig. 9 Collagen gel contraction assays. HDPCs-o (passage 3) at different cell numbers per well (**a**) or HDPCs-o and NIH 3T3, both at 3×10^4 cells/well (**b**), were mixed with rat tail or bovine collagen and plated in 96-well plates (100 μl/well) in duplicate. The diameters of the gels were measured at various time points (*error bars* SE). HDPCs-o and NIH 3T3a in rat-tail collagen (pH 7.0); NIH 3T3b in bovine collagen (pH 6.0)

on day 11 vs HDPCs-o on day 17, possibly explaining this growth discrepancy. NIH 3T3 did not grow extensively until later in the experimental period. Although a different pulp cell source and passages were used for the studies, the findings confirmed that pulp cell proliferation took place in collagen matrix.

Collagen gel contraction assays

In the studies of cell growth in collagen (see above), both HDPCs-d and HDPCs-o were equally potent on contracting collagen. To determine quantitatively the extent of collagen gel contraction affected by the concentration of pulp cells, various numbers of cells were mixed with collagen and cultured in 96-well plates. HDPCs-o were used in representative studies. At 1.5×10^5 cells/well and 5×10^4 cells/well, the collagen matrix contracted down to 30%–34% in 14–21 days, and at 10^4 cells/well down to 35% after 21 days, whereas the collagen gel without cells exhibited no contraction (Fig. 9a). It took 3, 5, and 15 days for collagen gel matrix in cultures with 1.5×10^5, 5×10^4, and 10^4 cells/well, respectively, to become half the size of their original diameter. Statistically significant contraction of collagen with 1.5×10^5 and 5×10^4 cells/well occurred on day 2 onward and for 10^4 cells/well group from day 8 and onward ($P<0.05$). The data indicated that the degree of contraction was proportionate to the number of cells cast in the gel.

The contraction property of pulp cells was compared with that of NIH 3T3 cells. We found that NIH 3T3 cells required slightly different pH values in the collagen gel to improve their adaptation to the environment. NIH 3T3 cells in collagen at pH 7.0 (NIH 3T3a group) grew much more slowly (as depicted in Fig. 8), and therefore only minimal contraction was observed (statistically significant only after 16 days; Fig. 9b). When collagen gel was prepared at pH 6.0, NIH 3T3 grew more extensively and caused significant contraction of the gel at a similar rate as pulp cells (NIH 3T3b vs pulp cells). Pulp cells grew well at pH 7.0 in both types of collagen gel used.

Discussion

The present study aimed to address several issues prerequisite to establishing a protocol for pulp tissue engineering and regeneration. First we wished to determine the difference, if any, of pulp cell phenotypes isolated by two methods, viz., enzyme digestion and the outgrowth method. The latter has been widely used by many investigators to study various aspects of pulp cell physiology (Chen et al. 2005; Hosoya et al. 1996; Nakao et al. 2004; Patel et al.

2003) or cell subpopulations potentially capable of differentiating into odontoblasts or mineral-forming cells in vitro (About et al. 2000; Couble et al. 2000; Saito et al. 2004; Tsukamoto et al. 1992). About et al. (2000) and Couble et al. (2000) have used the outgrowth method to obtain pulp cells and have been able to induce pulp cells to differentiate into odontoblast-like cells and generate dentin-like mineral structure in vitro. Tsukamoto et al. (1992) have isolated nodule forming pulp cells by using the outgrowth method. Gronthos et al. (2000) and Batouli et al. (2003), on the other hand, have applied enzyme digestion to isolate DPSCs and verified their differentiation into odontoblast-like cells and their production of dentin in vivo. Both methods appear to give rise to pulp cells containing odontoblast progenitor cells, although no report has established the in vivo capacity of pulp cells isolated by the outgrowth method to form dentin.

The advantage of isolating pulp cells via the outgrowth method perhaps is its convenience, although more time is needed to allow sufficient numbers of cells to migrate out of the tissue (up to 2 weeks). Fibroblasts in pulp tissue are reported to proliferate while migrating out into the culture dish (Vidic et al. 1972). We allowed pulp fragments to exhaust the outgrowth of cells by consecutively transferring explant fragments to a new well (24-well plate) approximately once a week until no further outgrowth was seen (up to 6 transfers). No live cells were observed in the exhausted tissue fragments observed by histological examination (data not shown), suggesting that perhaps all the cells migrated out into the dish or that the non-migrated cells disintegrated within the tissue. The digestion method supposedly releases all cells from the tissue; however, the process is technically difficult and inevitably some degree of cell damage and loss occurs. Nonetheless, this method allows different types of colony formation, and the single colonies may be further characterized for their stem cell characteristics, as previously reported (Gronthos et al. 2002). Our observation regarding the in vitro morphological categorizations of HDPCs-d outlined above agrees with that of Gronthos et al. (2000) in that DPSCs isolated by enzyme digestion form compact and loose types of colonies. Since this method releases all cell types in the pulp tissues, the observation of cells of different morphologies is unsurprising, as reported by other investigators (Gronthos et al. 2000; Nakashima 1991). To date, only a few types of adherent cells in pulp tissue are known to be capable of dividing; these include mesenchymal cells, pulp fibroblasts, endothelial cells, and pericytes. Shi and Gronthos (2003) have identified STRO-1-positive cells around blood vessels and perineurium in pulp tissues, and the majority of STRO-1-positive DPSCs expresses the pericyte marker, 3G5, suggesting that DPSCs may be pericytes. Shi and Gronthos (2003) have also found that the STRO-1 fraction represents approximately 6% of the total pulp cell population. Whether pulp cells derived from the outgrowth method constitute a similar or different percentage from that of STRO-1 cells requires further investigation.

The expression of DSPP, Cbfa1, and collagen types I and III in pulp is currently considered to be as follows: DSPP is dentin-specific and expressed mostly by odontoblast cells (MacDougall et al. 1997); the transcriptional factor Cbfa1 is expressed in mouse dental pulp clonal cell lines (Priam et al. 2005); both type I and III collagen are produced by pulp fibroblasts; collagen produced by odontoblasts is almost exclusively type I (Okiji 2002). Since pulp cells isolated by the different methods in our study exhibit differences in morphology and growth rate, they probably constitute different populations of cells. The passage of cells in vitro is considered a major factor in the loss of the capacity of stem cells to differentiate. Examination of the expression of DSPP, Cbfa1, and collagen types I and III of HDPCs-d and HDPCs-o from passages 1-5 represents a simple approach to detect differences, if they exist, in these cells. The conclusion that can be made from the results in Figs. 4 and 5 is that the two isolation methods indeed yield different populations of cells. The isolation of pulp cells with the potential to differentiate into odontoblasts is a critical step toward pulp tissue regeneration and new dentin formation. Therefore, further exploration is required to identify other differences in pulp cells isolated by these two methods, especially concerning changes occurring during passage. Although enzyme digestion appears to give rise to pulp cells of greater proliferation potential (a characteristic of energetic stem cells), additional investigations are required to demonstrate which method is superior to acquire pulp cells for the purpose of pulp tissue regeneration.

The range of pulp cell types produced in vitro can normally be observed when these cells are grown on the surface of culture dishes. Whether cells behave similarly when they are grown on the surface of human dentin in vitro is unclear. Batouli et al. (2003) have seeded DPSCs onto human dentin surfaces treated with citric acid and implanted them into immunocompromised mice. They have observed scattered reparative dentin-like structure deposited on the dentin surface. The reason that the entire dentin surface is not covered by newly formed dentin is unknown, as is whether the seeded DPSCs proliferate on the existing dentin. If pulp regeneration in a pulpless canal is possible clinically, then the canal space needs first to be cleansed chemically (at least with NaOCl and EDTA) and shaped mechanically during a routine endodontic procedure. To produce new dentin by the regenerated pulp tissue, the pulp stem cells would have to be able to attach to the treated dentin wall and subsequently proliferate and differentiate into odontoblasts in order to make new dentin. Our in vitro data of pulp cell growth on treated dentin surface indicate that pulp cells proliferate only to a limited extent on dentin, unlike their growth on culture dishes. Most interestingly, our findings indicate that HDPCs-d that attach to the dentin surface are capable of differentiating into cells of odontoblast morphology with processes (one major process per cell) extending into existing dentinal tubules (Fig. 7). This finding is different from that reported by Schmalz et al. (2001) who have shown that SV40 large T-antigen-transfected bovine pulp-derived

cells proliferate extensively on processed dentin and do not show odontoblast-like morphology after 14 days. Whereas recent reports suggest that newly differentiated odontoblasts extend their processes into existing dentinal tubules after pulp injury in rats in an in vivo setting (Nakakura–Ohshima et al. 2003; Ohshima et al. 2003), our finding is the first evidence for the phenomenon that human pulp cells of odontoblast-lineage differentiate into odontoblasts in vitro upon contact with dentin, even if it has been treated mechanically and chemically. The chemical treatment of dentin (e.g., by EDTA) may solubilize various noncollagenous dentin matrix components and growth factors, such as transforming growth factor-β1, which may have an inductive effect on the differentiation of odontoblast progenitor cells (Smith 2002). With regard to the promotion of pulp cell proliferation on dentin before their differentiation into odontoblasts, other avenues may be explored in the future (Nakao et al. 2004; Nakashima et al. 2004).

Although collagen is a commonly used matrix in which to grow cells in three-dimensions, several cell types (especially fibroblasts) are known to cause the contraction of collagen (Carlson and Longaker 2004; Grinnell 2000; Vernon and Sage 1996). To fabricate engineered mucosal tissue, methods are available to prevent collagen matrix contraction by gingival fibroblasts (Igarashi et al. 2003) or allow contraction to reach its maximum before the seeding of the keratinocyte layer (Okazaki et al. 2003). For pulp tissue engineering, it is important to know whether the collagen contraction caused by pulp fibroblasts is considerable enough to affect the outcome of pulp tissue regeneration. Collagen lattice contraction by pulp cells has been recently reported (Chan et al. 2005). Our data conform with the published report and demonstrate that pulp cells markedly cause the contraction of collagen with a reduction down to ~30%. Depending on the density of the cells in the matrix, the collagen gel shrinks to half of its original size by 3–15 days. NIH 3T3 cells also demonstrate a potent ability to contract collagen if given optimal growth conditions in collagen gels. It may be reasonable to predict that all fibroblastic cells can cause the contraction of a collagen matrix. We have observed that, after significant contraction, cells tend to migrate out of the collagen matrix. Additionally, our data have revealed that, although pulp cells proliferate more slowly in gels than in culture dishes, they still increase in number over time, thereby causing further contraction of the gel. Thus, if pulp tissue engineering protocols involve the mixing of pulp cells and collagen solution followed by the insertion of the mixture into a cleansed canal space in vivo to regenerate pulp tissue, then the mixture would shrink and could interfere with pulp tissue regeneration. Contraction-resistant scaffolds should therefore be tested or developed for pulp tissue engineering (Lee et al. 2001).

Before the development of a protocol to engineer and regenerate pulp tissue with differentiated odontoblasts capable of forming dentin, certain aspects of pulp cell behavior in vitro need to be investigated. Our present studies set the stage for further multi-directional investigations. Our data indicate that (1) different isolation methods give rise to different populations or lineages of pulp cells during in vitro passage, (2) direct contact of pulp cells with mechanically and chemically treated dentin may promote pulp cells to differentiate into odontoblasts with processes extending into dentinal tubules, and (3) collagen matrix alone may not be a suitable scaffold for pulp tissue engineering.

Acknowledgements We greatly appreciated the technical assistance of Alicia Thompson (Center for Electron Microscopy and Microanalysis) with the SEM analysis.

References

About I, Bottero MJ, Denato P de, Camps J, Franquin JC, Mitsiadis TA (2000) Human dentin production in vitro. Exp Cell Res 258:33–41

Alsberg E, Hill EE, Mooney DJ (2001) Craniofacial tissue engineering. Crit Rev Oral Biol Med 12:64–75

Batouli S, Miura M, Brahim J, T.W.Tsutsui, Fisher LW, Gronthos S, Robey PG, Shi S (2003) Comparison of stem-cell-mediated osteogenesis and dentinogenesis. J Dent Res 82:976–981

Bohl KS, Shon J, Rutherford B, Mooney DJ (1998) Role of synthetic extracellular matrix in development of engineered dental pulp. J Biomater Sci Polym Ed 9:749–764

Buurma B, Gu K, Rutherford RB (1999) Transplantation of human pulpal and gingival fibroblasts attached to synthetic scaffolds. Eur J Oral Sci 107:282–289

Carlson MA, Longaker MT (2004) The fibroblast-populated collagen matrix as a model of wound healing: a review of the evidence. Wound Repair Regen 12:134–147

Chan CP, Lan WH, Chang MC, Chen YJ, Lan WC, Chang HH, Jeng JH (2005) Effects of TGF-betas on the growth, collagen synthesis and collagen lattice contraction of human dental pulp fibroblasts in vitro. Arch Oral Biol 50:469–479

Chen S, Santos L, Wu Y, Vuong R, Gay I, Schulze J, Chuang HH, Macdougall M (2005) Altered gene expression in human cleidocranial dysplasia dental pulp cells. Arch Oral Biol 50:227–236

Couble ML, Farges JC, Bleicher F, Perrat–Mabillon B, Boudeulle M, Magloire H (2000) Odontoblast differentiation of human dental pulp cells in explant cultures. Calcif Tissue Int 66:129–138

Di Lenarda R, Cadenaro M, Sbaizero O (2000) Effectiveness of 1 mol L–1 citric acid and 15% EDTA irrigation on smear layer removal. Int Endod J 33:46–52

Drury JL, Mooney DJ (2003) Hydrogels for tissue engineering: scaffold design variables and applications. Biomaterials 24:4337–4351

D'Souza R (2002) Development of the pulpodentin complex. In: Hargreaves KM, Goodis HE (eds) Seltzer and Bender's Dental pulp. Quintessence, Carol Stream, pp 13–41

Froes JA, Horta HG, Silveira AB da (2000) Smear layer influence on the apical seal of four different obturation techniques. J Endod 26:351–354

Grinnell F (2000) Fibroblast-collagen-matrix contraction: growth-factor signalling and mechanical loading. Trends Cell Biol 10:362–365

Gronthos S, Mankani M, Brahim J, Robey PG, Shi S (2000) Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci USA 97:13625–13630

Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S (2002) Stem cell properties of human dental pulp stem cells. J Dent Res 81:531–535

Haas VR, Santos AR, Jr, Wada ML (2001) Behaviour of fibroblastic cells cultured in collagen I using the sandwich technique. Cytobios 106 (Suppl 2):255–267

Hosoya S, Matsushima K, Ohbayashi E, Yamazaki M, Shibata Y, Abiko Y (1996) Stimulation of interleukin-1beta-independent interleukin-6 production in human dental pulp cells by lipopolysaccharide. Biochem Mol Med 59:138–143

Igarashi M, Irwin CR, Locke M, Mackenzie IC (2003) Construction of large area organotypical cultures of oral mucosa and skin. J Oral Pathol Med 32:422–430

Lee KY, Mooney DJ (2001) Hydrogels for tissue engineering. Chem Rev 101:1869–1879

Lee KY, Alsberg E, Mooney DJ (2001) Degradable and injectable poly(aldehyde guluronate) hydrogels for bone tissue engineering. J Biomed Mater Res 56:228–233

MacDougall M, Simmons D, Luan X, Nydegger J, Feng J, Gu TT (1997) Dentin phosphoprotein and dentin sialoprotein are cleavage products expressed from a single transcript coded by a gene on human chromosome 4. Dentin phosphoprotein DNA sequence determination. J Biol Chem 272:835–842

Mooney DJ, Powell C, Piana J, Rutherford B (1996) Engineering dental pulp-like tissue in vitro. Biotechnol Prog 12:865–868

Nakakura–Ohshima K, Watanabe J, Kenmotsu S, Ohshima H (2003) Possible role of immunocompetent cells and the expression of heat shock protein-25 in the process of pulpal regeneration after tooth injury in rat molars. J Electron Microsc (Tokyo) 52:581–591

Nakao K, Itoh M, Tomita Y, Tomooka Y, Tsuji T (2004) FGF-2 potently induces both proliferation and DSP expression in collagen type I gel cultures of adult incisor immature pulp cells. Biochem Biophys Res Commun 325:1052–1059

Nakashima M (1991) Establishment of primary cultures of pulp cells from bovine permanent incisors. Arch Oral Biol 36:655–663

Nakashima M, Iohara K, Ishikawa M, Ito M, Tomokiyo A, Tanaka T, Akamine A (2004) Stimulation of reparative dentin formation by ex vivo gene therapy using dental pulp stem cells electrotransfected with growth/differentiation factor 11 (Gdf11). Hum Gene Ther 15:1045–1053

Ohshima H, Nakakura–Ohshima K, Takeuchi K, Hoshino M, Takano Y, Maeda T (2003) Pulpal regeneration after cavity preparation, with special reference to close spatio-relationships between odontoblasts and immunocompetent cells. Microsc Res Tech 60:483–490

Okazaki M, Yoshimura K, Suzuki Y, Harii K (2003) Effects of subepithelial fibroblasts on epithelial differentiation in human skin and oral mucosa: heterotypically recombined organotypic culture model. Plast Reconstr Surg 112:784–792

Okiji T (2002) Pulp as a connective tissue. In: Hargreaves KM, Goodis HE (eds) Seltzer and Bender's Dental Pulp. Quintessence, Carol Stream, pp 95–123

Onishi T, Kinoshita S, Shintani S, Sobue S, Ooshima T (1999) Stimulation of proliferation and differentiation of dog dental pulp cells in serum-free culture medium by insulin-like growth factor. Arch Oral Biol 44:361–371

Park SH, Hsiao GY, Huang GT (2004) Role of substance P and calcitonin gene-related peptide in the regulation of interleukin-8 and monocyte chemotactic protein-1 expression in human dental pulp. Int Endod J 37:185–192

Patel T, Park SH, Lin LM, Chiappelli F, Huang GT (2003) Substance P induces interleukin-8 secretion from human dental pulp cells. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 96:478–485

Priam F, Ronco V, Locker M, Bourd K, Bonnefoix M, Duchene T, Bitard J, Wurtz T, Kellermann O, Goldberg M, Poliard A (2005) New cellular models for tracking the odontoblast phenotype. Arch Oral Biol 50:271–277

Saito T, Ogawa M, Hata Y, Bessho K (2004) Acceleration effect of human recombinant bone morphogenetic protein-2 on differentiation of human pulp cells into odontoblasts. J Endod 30:205–208

Schmalz G, Garhammer P, Schweiki H (1996) A commercially available cell culture device modified for dentin barrier tests. J Endod 22:249–252

Schmalz G, Schuster U, Thonemann B, Barth M, Esterbauer S (2001) Dentin barrier test with transfected bovine pulp-derived cells. J Endod 27:96–102

Shi S, Gronthos S (2003) Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res 18:696–704

Smith AJ (2002) Dentin formation and repair. In: Hargreaves KM, Goodis HE (eds) Seltzer and Bender's Dental Pulp. Quintessence, Carol Stream, pp 41–63

Tsukamoto Y, Fukutani S, Shin–Ike T, Kubota T, Sato S, Suzuki Y, Mori M (1992) Mineralized nodule formation by cultures of human dental pulp-derived fibroblasts. Arch Oral Biol 37:1045–1055

Vacanti CA, Langer R, Schloo B, Vacanti JP (1991) Synthetic polymers seeded with chondrocytes provide a template for new cartilage formation. Plast Reconstr Surg 88:753–759

Vacanti JP (2003) Tissue and organ engineering: can we build intestine and vital organs? J Gastrointest Surg 7:831–835

Vernon RB, Sage EH (1996) Contraction of fibrillar type I collagen by endothelial cells: a study in vitro. J Cell Biochem 60:185–197

Vidic B, Chen R, Taylor JJ (1972) Functional behavior and structural modification of pulpal fibroblasts cultured in vitro. Archs Arch Anat Histol Embryol 55:51–73

Zhu YK, Umino T, Liu XD, Wang HJ, Romberger DJ, Spurzem JR, Rennard SI (2001) Contraction of fibroblast-containing collagen gels: initial collagen concentration regulates the degree of contraction and cell survival. In Vitro Cell Dev Biol Anim 37:10–16