

K. KADAR¹, M. KIRALY¹, B. PORCSALMY¹, B. MOLNAR¹, G.Z. RACZ¹, J. BLAZSEK¹,
K. KALLO¹, E.L. SZABO¹, I. GERA³, G. GERBER², G. VARGA¹

DIFFERENTIATION POTENTIAL OF STEM CELLS FROM HUMAN DENTAL ORIGIN - PROMISE FOR TISSUE ENGINEERING

¹Department of Oral Biology, Semmelweis University, Budapest, Hungary; ²Department of Anatomy, Histology and Embriology Semmelweis University, Budapest, Hungary; ³Department of Periodontology, Semmelweis University, Budapest, Hungary

Recent studies have revealed the existence of stem cells in various human tissues including dental structures. We aimed to establish primary cell cultures from human dental pulp and periodontal ligament, to identify multipotential adult stem cells in these cultures, and to study the differentiation capacity of these cells to osteogenic and to neuronal fates. Dental pulp and the periodontal ligament were isolated from extracted human wisdom teeth. The extracellular matrix was enzymatically degraded to obtain isolated cells for culturing. Both dental pulp and periodontal ligament derived cultures showed high proliferative capacity and contained a cell population expressing the STRO-1 mesenchymal stem cell marker. Osteogenic induction by pharmacological stimulation resulted in mineralized differentiation as shown by Alizarin red staining in both cultures. When already described standard neurodifferentiation protocols were used, cultures exhibited only transient neurodifferentiation followed by either redifferentiation into a fibroblast-like phenotype or massive cell death. Our new three-step neurodifferentiation protocol consisting of (1) epigenetic reprogramming, then (2) simultaneous PKC/PKA activation, followed by (3) incubation in a neurotrophic medium resulted in robust neurodifferentiation in both pulp and periodontal ligament cultures shown by cell morphology, immunocytochemistry and real time PCR for vimentin and neuron-specific enolase. In conclusion, we report the isolation, culture and characterization of stem cell containing cultures from both human dental pulp and periodontal ligament. Furthermore, our data clearly show that both cultures differentiate into mineralized cells or to a neuronal fate in response to appropriate pharmacological stimuli. Therefore, these cells have high potential to serve as resources for tissue engineering not only for dental or bone reconstruction, but also for neuroregenerative treatments.

Key words: *human, dental pulp, periodontal ligament, stem cell, STRO-1, proliferation, osteogenic differentiation, neuronal differentiation*

INTRODUCTION

Stem cell research has become a promising field for tissue regeneration and implementation of regenerative medicine. Since the discovery and characterization of multipotent mesenchymal stem cells from bone marrow, similar populations from other tissues have now been characterized. Postnatal stem cells have been isolated from a variety of tissues including bone marrow, brain, skin, skeletal muscle and the gastrointestinal tract (1-3). Recent studies have revealed the presence of adult stem cells in tissues of dental origin as well (4-6). Namely, primary cell cultures containing progenitor cells originating from both adult and deciduous dental pulp as well as periodontal ligament were described (4-6). Recently, an extraordinary plasticity of postnatal stem cells has been suggested. Bone marrow stem cells may contribute to muscle, liver, and neuronal tissue formation (5-8) and neural stem cells may contribute to blood and skeletal muscle regeneration (9-11). To utilize this potential, it is necessary to gain further insight into the characteristics of postnatal stem cells of dental origin and examine their full developmental potential first *in vitro* then *in vivo*.

Since the stem cell cultures of dental origin exhibit mesenchymal stem cell characteristics (4-6), one of the most plausible direction for differentiation and potential utilization of these cells is the osteogenic one. Indeed, one important feature of both pulp and periodontal cells is their mineralization potential in response to appropriate pharmacological induction (4-6). Cells can be induced *in vitro* to differentiate into cells of osteogenic/odontogenic phenotype, characterized by polarized cell bodies and accumulation of mineralized nodules (12-14). Nevertheless, the exact molecular signaling mechanism for this transition, and also the interaction of various pathways being involved is not completely understood.

The dental pulp and the periodontal ligament have also been suggested to harbor cells that are able to differentiate into neuronal direction (5, 15-21). Neural progenitors may have the potential for neurogenesis similar to that of embryonic stem cells that are being investigated for therapeutic purposes (22). The availability of adult neural progenitor candidates could advance the perspective of cell therapy for neural diseases. Preceding studies demonstrated that when transplanted into adult rodent brain, dental pulp stem cells (DPSCs) infiltrated into the host

nerve tissue, and expressed neurospecific markers (5, 18). In addition, recently a pioneer study demonstrated that DPSCs can be differentiated into neuronal-like cells. Application of Neurobasal Medium supplemented with differentiation factors (15), resulted in an incomplete neuronal differentiation of these cells, since only voltage gated sodium channels could be detected without the presence of voltage gated potassium channels which are also regarded as a basic criterion for functional neuronal cell identification (15). Other recently developed experimental protocols using various induction mixtures for pharmacological induction of neuronal differentiation also resulted in partial results, achieving only reversible differentiation followed by either dedifferentiation (23) or massive cell death (24). Therefore, further studies are needed to better understand the factors involved in neuronal differentiation.

The purpose of the present study was to establish primary cell cultures from human dental pulp and periodontal ligament and to identify multipotential adult stem cells in these cultures. Then, using optimized pharmacological protocols, we compared the potential of pulp and periodontal cultures to form mineralized tissues and to undergo neuronal differentiation.

MATERIALS AND METHODS

Isolation and cell culture

Our protocol to isolate and culture dental pulp stem cells is based on a procedure described previously (4), with some modifications. In brief, normal human impacted third molars were collected from adults (18-26 years of age) at the Department of Periodontology, Semmelweis University, under approved ethical guidelines set by the Ethical Committee of the Hungarian Medical Research Council. Tooth surfaces were cleaned and the periodontal tissue was removed with a sterile scalpel and was collected. The tooth was cut around the cemento-enamel junction by sterile dental fissure burs to expose the pulp chamber, and the pulp tissue was removed from the crown and root. Both pulp tissue and periodontal tissue were then separately digested in a solution of collagenase type I (3 mg/ml, Sigma) and dispase (4 mg/ml, Roche) for 1 h at 37°C. Single-cell suspensions were obtained by passing the cells through a 70 µm strainer (Falcon) and were seeded into 6-well plates (Costar) in alpha modification of Eagle's medium (α -MEM, GIBCO/BRL) supplemented with 15% (periodontal tissue) or 20% (pulp tissue) Fetal Bovine Serum (FBS, GIBCO/BRL), 100 µM L-ascorbic acid 2-phosphate (Sigma), 2 mM L-glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin (GIBCO/BRL), and then incubated at 37°C in 5% CO₂. To assess colony-forming capability, 14 day old cultures were fixed with 4% paraformaldehyde, and then stained with 2% Giemsa. Aggregates of 50 cells were scored as colonies.

Cell viability and cell toxicity studies

To test the effect of serum and also serum-free conditions on culture growth, dental pulp stem cells (DPSCs) and periodontal ligament stem cells (PDLSCs) were cultured in 96-well plates for 24 hours. In each well 3 x 10³ DPSC cells or 5 x 10³ PDLSC cells were grown in their regular media for 24 hours. Afterwards, cells were serum-starved for another 24 hours. Then, 15% (PDLSC) or 20% (DPSC) FBS containing medium, or serum-free medium (control) was added for 24 hours. Thereafter, 100 µl MTT solution (0.2 mg/ml, Sigma) diluted in α -MEM was added into each well until formazane crystal formation occurred. 100 µl DMSO (99.5%, dimethyl-sulfoxid) was added into wells

to dissolve formazane crystals. Then the intensity of staining was determined by a microplate reader (Biorad, Model 3550) at 595 nm (measurement wavelength) and 650 nm (reference wavelength). Under these circumstances, the level of optical density is proportional to the number of living cells in the culture. The proliferative effect was expressed as a ratio between optical density of treated cells and serum-free cultured control cells and given in percent.

Osteogenic induction

Osteogenic differentiation was induced by modifications of a previously reported protocol (25). In brief, DPSCs and PDLSCs were cultured with 1% FBS, 100 mg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, 10⁻⁸M dexamethazone, 50 mg/ml L-ascorbic acid 2-phosphate, 10 mmol/l β -glycerophosphate in α MEM for 20 days without passaging. The medium was replaced twice a week. After 3 weeks of treatment calcium accumulation was detected by 2% Alizarin red S (pH 4.2, buffered with ammonium hydroxide) staining. Similar culture media without dexamethazone and β -glycerophosphate was used as control condition.

Neuronal induction

For neuronal differentiation, cultured morphologically homogeneous DPSCs and PDLSCs, (passage 1-4) were plated (~2x10⁴ cells/well) into a 24 well plate containing poly-L-lysine coated glass coverslips. After 24 hours, cells were treated with 3 different protocols:

Protocol 1. Cells were differentiated as previously described by Scintu *et al.* (23) with 10 ng/ml FGF-1 (R&D, Minneapolis, MN), 200 nM 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma, St Louis, USA), 250 µM IBMX (Sigma) and 50 µM forskolin (Sigma), in Dulbecco's modified Eagle's medium/F12 1:1 (DMEM/F12) (Sigma) supplemented with ITS Liquid Media Supplement (Sigma). The cells were fixed for immunocytochemistry right before and 24 h post-induction.

Protocol 2. is also based on a method recently reported by Choi *et al.* (24). Cells were preinduced for 1 day with DMEM/F12, with 20% FBS, and 10 ng/ml basic fibroblast growth factor (bFGF; Sigma, St.Louis, MO). The preinduction medium was removed, cells were washed with phosphate-buffered saline (PBS) and then changed to serum-free induction medium that consisted of DMEM containing 2% DMSO, 200 µM BHA, 25 mM KCl, 2 mM valproic acid, 10 µM forskolin, 1 µM hydrocortisone and 5 µg/ml insulin (Sigma). The cells were fixed for immunocytochemistry right before and 24 h post-induction.

Protocol 3. a three-step differentiation method was developed in our own laboratory since Protocols 1 and 2 did not yield satisfactory results. DPSCs or PDLSCs were seeded onto poly-L-lysine coated glass coverslips in DMEM/F12, 2.5% FBS, 100 mg/ml streptomycin, and 100 U/ml penicillin, and cultured for 24 h. Step 1: epigenetic reprogramming was performed using 10 mM 5-azacytidine in DMEM/F12 containing 2.5% FBS and 10 ng/ml bFGF for 48 h. Step 2: neural differentiation was induced by exposing the cells to 250 mM IBMX, 50 mM forskolin, 200 nM TPA, 1 mM dbcAMP, 10 ng/ml bFGF, 10 ng/ml NGF and 30 ng/ml NT-3, supplemented with ITS Liquid Media Supplement in DMEM/F12 for 3 days. Step 3: at the end of the neural induction treatment, cells were washed with PBS, and then neuronal maturation was performed by maintaining the cells in Neurobasal A media supplemented with 1 mM dbcAMP, 1% N2, 1% B27, and 30 ng/ml NT-3 for 3-8 days. Solutions

were freshly prepared immediately prior to use. The cells were fixed for immunocytochemistry before treatment, on the first day neuronal induction (step 2) and on the third day of maturation (step 3).

Immunocytochemistry

To identify the mesenchymal stem cell marker STRO-1 in our cultures, cells were grown on glass coverslips in 24-well plates (Costar) (5×10^4 cells per well) and fixed with 4% PFA in PBS for 20 min. To block non-specific binding, fixed cultures were incubated in PBS containing 7.5% FBS for 90 min and incubated with an anti-STRO-1 primary antibody (1/200, a generous gift from Prof Richard Oreffo, University of Southampton, Southampton, UK) overnight at 4°C. Subsequently, the cells were incubated with Alexa 488 conjugated goat anti-mouse IgG (1:1000, Molecular Probes) for 1 hour. Nuclei were counterstained with 10 mg/ml bisbenzimidazole (Sigma) for 30 minutes.

To evaluate protein expression during differentiation experiments, cells grown on poly-L-lysine-coated glass coverslips were fixed with 4% PFA in PBS for 20 min at room temperature (RT), then 0.1% Triton X-100 (in PBS) was added for 8 min to permeabilize them. Fixed cultures were incubated in PBS containing 4% bovine serum albumin (BSA; 90 min at RT) to block non-specific binding, then reacted with primary antibodies at 4°C overnight. Antibodies were diluted in 4% BSA as follows: anti-NSE 1/200, anti-NF-M 1/200. IgG anti-mouse and anti-rabbit Alexa Fluor 488 conjugated (Molecular Probes) secondary antibodies were diluted 1/750 and applied for 1 h at RT. Nuclei were counterstained with 10 mg/ml bisbenzimidazole (Sigma) for 30 minutes.

Labeled preparations were examined by a fluorescent microscope (Nikon Eclipse E600, Nikon Instruments), and images were captured with a cooled CCD camera (SPOT RT Color 2000, Diagnostic Instruments) connected to a PC running an image acquisition software (SPOT Advanced, Diagnostic Instruments). Adobe Photoshop was used to merge the digitized images of bisbenzimidazole and specific staining.

Real-time PCR

Total RNA from DPSCs and PDLSCs was isolated using an RNeasy Plus Micro Kit (Qiagen) with on-column DNase digestion. The concentration of the RNA was determined by the Ribogreen method (Invitrogen). The integrity of the RNA was verified by electrophoresis on a 1% agarose gel and 200 ng total RNA was used per sample for cDNA synthesis, using random primers (High-Capacity cDNA Archive Kit, Applied Biosystems) in a total volume of 50 μ l. For quantitative PCR amplification, 5% of the cDNA synthesis reaction was used with real time PCR primers and a target-specific fluorescence probe (FAM-labeled MGB probe). The probes and primers were selected from the Applied Biosystem Assay on Demand database for the specific markers vimentin (VIM) and neurospecific enolase (NSE), and for the human acidic ribosomal phosphoprotein P0 (RPLP0), which was used as an internal control. Universal Mastermix (Roche Diagnostics) containing AMP-erase was used for amplification in a total volume of 20 μ l. For detection of fluorescence signal during the PCR cycles, a (StepOne® Real-Time PCR System, Applied Biosystem) was used with the default setting (50°C for 2 min, 95°C for 10 min, 45 cycles: 95°C for 15 s, 60°C for 1 min). Each treatment was repeated five times and each sample was measured in duplicate. Changes in gene expression levels were estimated by calculating the relative expression values normalized to the RPLP0 level from the same sample.

Statistical analysis

Data are presented as means \pm S.E.M. For statistical comparisons, analysis of variance was followed by Bonferroni post-hoc test (Instat, GraphPad Software).

RESULTS

Isolation and primary culture

As it has been described, osteogenic stem cells can be isolated from aspirates of bone marrow by their ability to adhere to a plastic surface, and with appropriate stimulation these cells start to proliferate. Under these circumstances, each colony originates from a single progenitor cell and displays a wide variation in cell morphology and growth potential. In the present study we were able to show the ability of both pulp-derived and periodontal ligament-derived cells to form adherent clonogenic cell clusters of fibroblast-like cells, similar to those recorded for other mesenchymal stem-cell populations. These colony-forming cell populations, which we termed dental pulp stem cells (DPSC) and periodontal ligament stem cells (PDLSC), had high proliferation rate, as demonstrated by the doubling of the cell number during culture in about two days. For this reason, cells were passaged once a week until they reached confluency. At this time cells were passaged 1:4. Cell cultures successfully established from pulp tissue were cultivated for up to 20 passages.

Both DPSC (*Fig. 1*) and PDLSC cultures showed typical fibroblast-like morphology and high clonogenic activity similar to the progeny of human bone marrow colony forming units. A fraction of the cells in DPSC (*Fig. 1D*) and PDLSC cultures expressed the cell surface molecule STRO-1, a mesenchymal stem cell marker, which is also present in bone-marrow derived and periodontal ligament derived stem cell cultures. STRO-1 immunoreactivity gradually decreased with increasing passage numbers, but 6-8% of the cells were still STRO-1 positive even at higher passage numbers.

Cell viability studies

As mentioned above, both DPSC and PDLSC containing primary cultures of pulp cells were growing continuously and had to be passaged frequently because of the fast doubling rate. To test the importance of serum in the test medium, cells were serum-starved for 24 h and then either received 20% FBS or were left in serum-free α -MEM medium. To validate our assay system, as an internal control, we plated only half of the cells into some wells. Our data revealed that FBS stimulated cell proliferation compared to serum-free controls. When 50% less cells were initially plated, MTT assay also showed about 40% less optical density than in control after 24 h incubation. DPSC cells showed a 209% \pm 9% proliferation rate in 20% FBS supplemented medium relative to the control without FBS. PDLSC cells grown in medium with 15% FBS had a proliferation rate of 142% \pm 8% compared to control.

Osteogenic differentiation

In these experiments DPSC and PDLSC (*Fig. 1E-F*) cultures were grown in the presence of osteogenic differentiation cocktail consisting of dexamethazone, L-ascorbic acid 2-phosphate and β -glycerophosphate. Under these conditions cultures uniformly demonstrated the capacity to form Alizarin red S positive condensed nodules with high calcium content. The deposits were

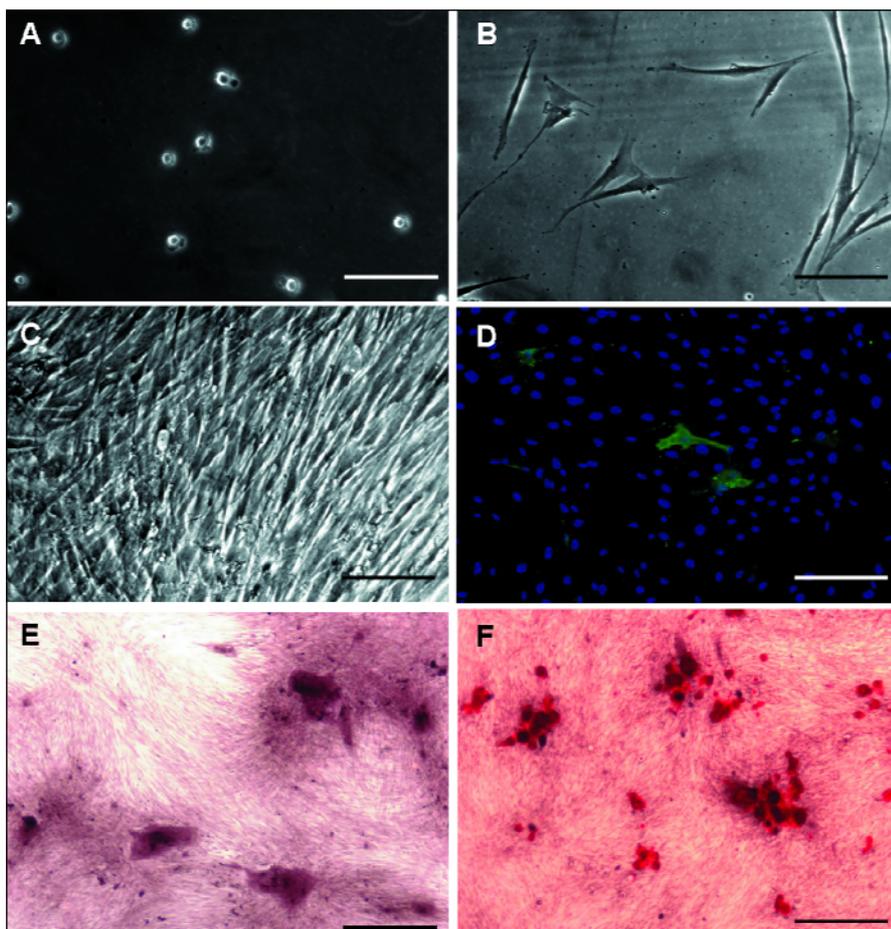


Fig. 1. DPSC culture (p2) STRO-1 immunoreactivity, cell morphology, during culture, and osteogenic differentiation. (A) Rounded DPSCs before attaching to the plastic surface directly after isolation, (B) after attaching to the surface cells showed flat, spindle-shaped, fibroblast-like morphology. (C) Confluent DPSC culture after 3 weeks. (D) Immunocytochemistry demonstrated that cultured DPSCs expressed STRO-1, a mesenchymal stem cell marker (E) DPSCs produced mineralized deposits under osteogenic conditions as detected by 2% Alizarin Red S staining. (F) Control staining of non-induced DPSCs. The length of scale bars indicate 100 μm .

sparingly scattered throughout the adherent layer as single mineralized zones. Control cultures formed adherent layers without any sign of calcium deposition. Thus, this observation confirms the previous findings that DPSCs and PDLSCs are capable of differentiation to mineralized tissue *in vitro* in response to appropriate pharmacological stimulation.

Neuronal differentiation

1. Partial differentiation induced by Protocols 1 and 2

When PDLSC and DPSC cells were treated using Protocol 1 with a mixture containing substances such as TPA, IBMX and forskolin, the morphology of cells observed by phase contrast microscopy changed rapidly (*Fig. 2A*, data shown for DPSC). After 4 hours of treatment initiation, the cytoplasm of the cells retracted toward the nucleus in many cells, taking a more spherical shape and extending processes. The percentage of cells with modified morphology decreased within 24 hours. When differentiation factors were removed from the medium, cell morphology reverted to the original. Following the application of Protocol 2 (bFGF, KCl, forskolin, DMSO and BHA) neuronal morphology changes were temporary, followed by an irreversible distraction of the culture: after 48 hours, the majority of cells were seen to have a rounded morphology followed by the death of almost the entire culture, except for a few spindle-shaped cells expressing none of the neuronal markers investigated by us (*Fig. 2B*, data shown for DPSC). These morphological changes were very similar for DPSC and PDLSC after both treatments.

2. Robust neuronal differentiation induced by Protocol 3

While Protocol 1 and Protocol 2 resulted in short term and reversible neuronal differentiation or even death of the cells after 48 h, Protocol 3, our three step differentiation procedure over 9 days resulted in a robust differentiation of both pulp cultures and periodontal cells towards neural lineages in essentially all surviving cells that initially showed the characteristics of dental fibroblasts. The originally fibroblast-like PDLSC and DPSC cells (*Fig. 2C*, data shown for DPSC) become more rounded after pretreatment with 5-azacytidine and bFGF for 48 h (epigenetic reprogramming). As early as after 2 h of treatment with the inducing mixture, PDLSCs and DPSCs grew processes and started moving towards the high cell-density areas. During the 3 days of induction, cells anchored their position in the network structure, the previously developed processes disappeared, and their morphology reverted to flat and round cell shapes observed during earlier stages of differentiation. In the final maturation step, cells diverging radially from the centers began to grow neurite-like processes. After 9 days of differentiation, the vast majority of cells, derived from either the dental pulp or the periodontal ligament, displayed complex neuronal morphology, expressing both bipolar and stellate forms. However, a small portion of cells retained their flat shape, and were attached beneath the processes of the neuronal cells. These elements are presumably committed towards glial fates, or serve as stanchions for the developing neuronal cells. Therefore, they might be indispensable for neuronal survival.

The time-dependent changes of neuronal marker gene expression in DPSC and PDLSC cultures undergoing neural development were evaluated by real time PCR (*Fig. 4*). Total

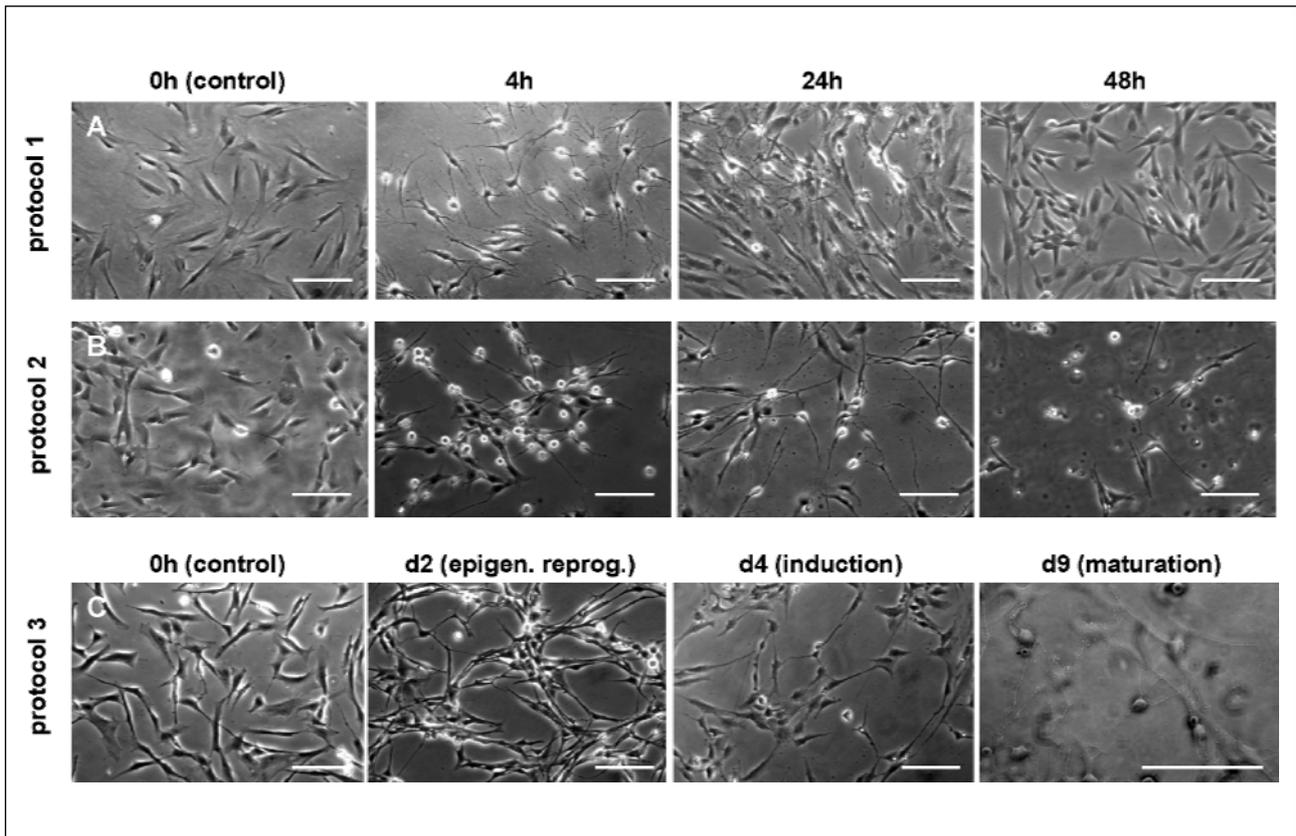


Fig. 2. Morphological changes during neuronal differentiation to different protocols in DPSCs. (A) Control DPSCs had spindle-shaped morphology on poly-L-lysine coated plastic surface. After culturing for 4 hours with Protocol 1, cytoplasm was retracted towards the nucleus, resulting more rounded cell body with long, extending processes. The ratio of cells with modified morphology decreased by 24 hours. When differentiation factors were removed from medium, cell morphology reverted to the original shape (48 hours). (B) Control DPSCs had spindle-shaped morphology. After treatment for 4 hours with Protocol 2, cells showed similarly to protocol 1 rounded cell body with long, extending processes. However, the ratio of cells with modified morphology has not changed by 24 hours. Extensive cell death were observed by 48 hours, the surviving cells does not show neuronal morphology. (C) Control DPSCs had spindle-shaped morphology on poly-L-lysine coated plastic surface. After 2 days of epigenetic reprogramming, cells formed clusters. After 4 days (2 days of induction), cells had morphological features typical of neurons, showing complex neuronal processes. After 9 days (4 days of maturation), most of the cells displayed either multi- or bipolar forms. The length of scale bars indicate 100 μ m.

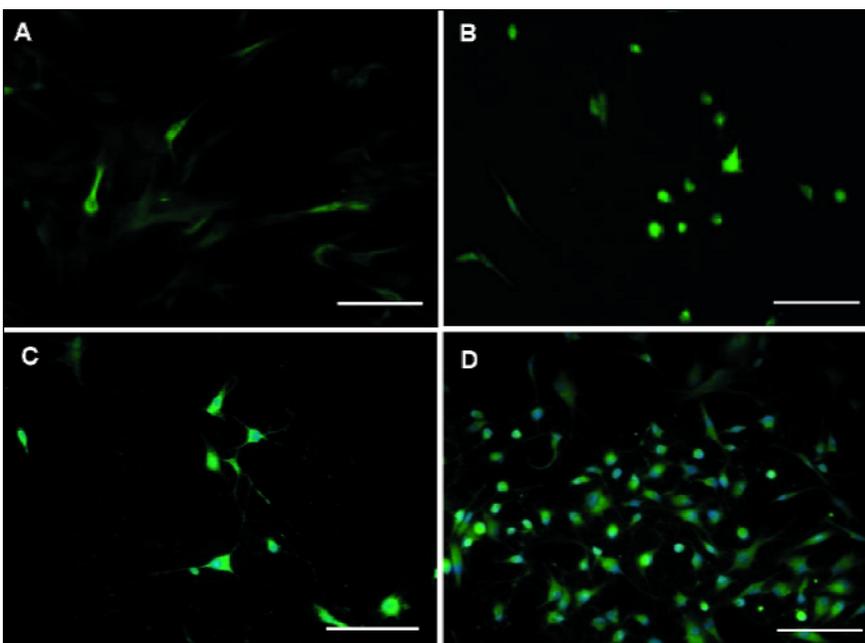


Fig. 3. Protein expression patterns of neuronal markers during neuronal differentiation. (A) Non-induced PDLSCs stained with anti-NFM, (B) PDLSCs stained with anti-NFM after 1 day of induction (step 2), (C) DPSCs stained with anti-NFM, and (D) DPSCs stained with anti-NSE after 8 days of treatment (maturation, step 3). Nuclei were visualized by bisbenzimidide. The length of scale bars indicate 100 μ m.

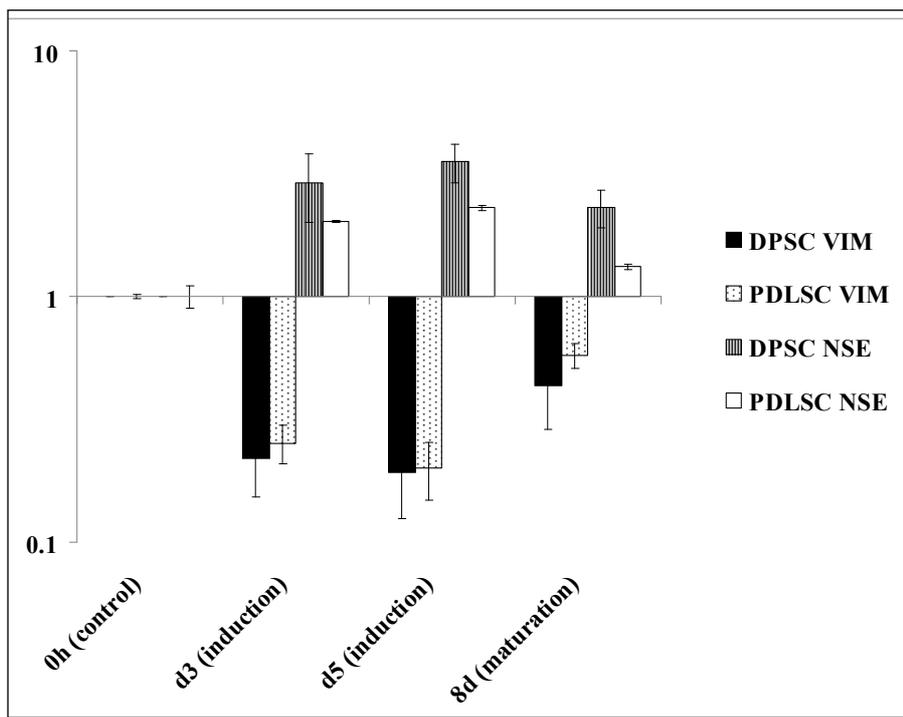


Fig. 4. mRNA expression pattern of mesenchymal, neuronal markers in DPSCs and PDLSCs during neuronal differentiation with protocol 3. Total RNA of DPSCs isolated from five independent normal human donors was used. RNA was harvested at four time points during the differentiation of each sample: from non-induced DPSCs (0 day), after the first day of induction (3rd day), on the third day of induction (5th day) and on the third day of maturation (8th day). Target gene expressions were normalized to RPLP0 housekeeping gene expression levels and evaluated as fold change relative to the non-induced sample. mRNA expression of the mesenchymal vimentin appeared to be significantly suppressed by the treatment, while the mRNA expression of the neuron specific glycolytic enolase NSE was significantly elevated by the treatment. DPSCs and PDLSCs showed similar gene expression pattern. Data are reported as mean \pm S.E.M.

RNA was harvested at four different time points during this period: at time 0 (noninduced control DPSCs and PDLSCs), on the first day of induction (d3), on the third day of induction (d5), and after three days of maturation (d8). The expression of each target gene was normalized to that of the RPLP0 housekeeping gene, and expressed as fold change relative to the noninduced sample. DPSCs and PDLSCs showed a very similar expression pattern during neuronal differentiation treatment. There was a sharp decrease in the expression of the mesenchymal marker vimentin in response to neurogenic induction. This very striking decrease became less pronounced during maturation. The expression of the neuronal marker NSE progressively increased under maturing conditions and remained increased during maturation.

The protein expression pattern investigated by immunocytochemistry corresponded well to the gene expression data. Non-induced DPSCs and PDLSCs (Fig. 3A) showed weak, nonspecific expression pattern of NFM. The neuronal induction resulted in an increased proportion of cells with an expression pattern specific to mature neurons in both DPSC and PDLSC (Fig. 3B) cultures. After 8 days cells also showed positive staining for NFM and NSE (Fig. 3C-D), markers typically expressed by mature neurons.

DISCUSSION

Recent studies have suggested that human bone marrow and dental pulp as well as periodontal ligament tissue contain postnatal stem cells that are capable of differentiating into various cell types including osteoblasts, odontoblasts, cementoblasts, adipocytes and neuronal cells (4-6). These stem cells were characterized as STRO-1 positive progenitors (5, 6, 26). In the present study we established primary cultures from human adult dental pulp and periodontal ligament and confirmed that these cultures contain DPSCs and PDLSCs, respectively, which are similar to other mesenchymal stem cells in that they are highly clonogenic and show expression of the STRO-1 mesenchymal progenitor cell marker.

The mechanisms controlling the development of teeth are largely unknown (27), in particular with respect to how craniofacial components including bone and soft tissues surrounding teeth, participate in the process of tooth development. DPSCs clearly have the ability to regenerate dentin, at least in experimental animals (28, 29) and therefore have a high potential for tooth regeneration as odontoblast progenitors. However, based on current information, DPSCs and PDLSCs may have a broader capacity for differentiation than originally thought (30). Growing evidence suggests that they are able to differentiate into several different cell types (4-6, 30). This phenotypic conversion may also be utilized in the future for applications beyond dentistry such as in studies on fat tissue generation and neuronal regeneration. Our study provides evidence that DPSCs represent a population of postnatal stem cells capable of extensive proliferation. Teeth may be an ideal resource of stem cells to repair damaged dental structures, induce bone regeneration, and possibly to treat neural tissue injury or degenerative diseases. For this reason, the establishment of DPSC cultures in our laboratory as well as in others opens a very promising area in cell based therapies for both modern dentistry and its related fields.

Isolation and maintenance of cells require special culture conditions. Most of the studies are still conducted using fetal bovine serum (FBS) as a supplement to culture media since cells are unable to proliferate without essential growth factors, hormones and nutrients that are present in the serum (31-33). Serum-free cell culture would represent an adequate alternative, but it requires careful adaptation of sensitive cell lines and often results in lower proliferation rates. As the use of special serum-free conditions has become state of the art in human tissue engineering applications, it is important to know how cells behave in serum-free environment compared to FBS-supplemented conditions. Nowadays serum-free medium is also available and there are a number of successful attempts to use them (21, 34-36). In our experiments re-stimulation with serum for 24h resulted in a much higher rate of proliferation compared to serum-free conditions. However, our data also indicate that a considerable number of cells survive short term serum-free conditions. Our data

emphasize that optimizing conditions may soon lead to successful serum-free culture of DPSCs and PDLSCs, which are potentially applicable for human transplantation.

The osteogenic differentiation cocktail that we used induced similar and well reproducible mineralization in both DPSC and PDLSC cultures. This pharmacological manipulation is based on simultaneous administration of dexamethasone, L-ascorbic acid (vitamin C) and β -glycerophosphate. The osteogenic differentiation potential of mesenchymal stem cells *in vitro* and *in vivo* has been well documented in a variety of studies. Several recent studies reported that DPSCs and PDLSCs were also capable of osteogenic differentiation (4, 16). In our experimental conditions, the use of the above described mixture resulted in the accumulation of insoluble calcium deposits. Dexamethasone, a synthetic steroid, has been shown to promote osteogenic differentiation of both embryonic (37, 38) and adult (39) stem cells. It is believed to be a crucial regulator of BMSC osteogenic differentiation (40). The precise mechanism of its action, however, is still obscure. Recently dexamethasone has been shown to inhibit osteoblastic differentiation through the repression of BMP-2 expression (41). However, most studies argue that DEX *in vitro* enhances osteoblastic differentiation, alkaline phosphatase (ALP) activity and bone mineralization. Thus, whether dexamethasone inhibits or promotes osteoblastic differentiation and bone formation in MSCs remains controversial, and the biological mechanism and signaling pathway by which DEX affects osteoblastic differentiation remains obscure (42).

Dexamethasone is usually used in combination with L-ascorbic acid (vitamin C) and β -glycerophosphate. The latter one clearly serves as a phosphate donor that is necessary to build up the mineral phase. The importance of ascorbic acid in the maintenance of normal extracellular matrix has been known for many years. It is required as a cofactor for prolyl-4-hydroxylase, an enzyme that hydroxylates proline, an important reaction that is necessary for the synthesis of collagen, a crucial protein component of all mesenchymal mineralizing matrices (43). In addition, ascorbate has been shown to stimulate proliferation and alkaline phosphatase expression and activity in osteoblastic cells (44, 45). Recently a novel mechanism of action of ascorbic acid was also suggested (46): increased type II and type X collagen secretion in the presence of ascorbate resulted in increased interactions with annexin V, stimulation of annexin V-mediated Ca^{2+} influx and, as a consequence, increased alkaline phosphatase expression and activity, leading to enhanced mineralization.

In our neurodifferentiation experiments, when we applied Protocol 1 both DPSC and PDLSC cells exhibited a transient neurodifferentiation. Very similar observations were made following treatment of human BMSCs with the PKC activator TPA, the phosphodiesterase inhibitor IBMX, the adenylate cyclase activator forskolin and bFGF: stimulation of the cells resulted in drastic morphological changes accompanied by the expression of neural markers such as neurofilaments, N-tubulin, NSE and GFAP (23). However, their expression was transient, and the cells reverted to the original fibroblastic BMSCs within 48 hours (23). Altogether, these findings indicate that the neurodifferentiation potential of Protocol 1 is limited. When we used Protocol 2, based on another recently described method (24), again we observed only transient neurodifferentiation followed by the death of most of the cells. This transient change followed by the death of the cells is most probably due to the extremely high, 25 mM K^+ concentration. Therefore, when we further developed our differentiation protocol, normal K^+ concentration was used.

Our newly developed neurodifferentiation method, Protocol 3, is based on the elements of previously used methods in a special temporo-spatial arrangement. The first element aims at dedifferentiating the cells by the application of 5-azacytidine, a

cytidine analog where nitrogen replaces a carbon at the 5th position of the pyrimidine ring. DNA methylation is known to be essential for cell differentiation as it modulates the transcription of genes in two ways. First, DNA methylation prevents the transcriptional regulator proteins from binding to the appropriate sequence, and second, methylated DNA can be bound by methyl-CpG-binding domain proteins, recruiting additional chromatin remodeling proteins forming compact, inactive silent chromatin. Treatment with 5-azacytidine causes hemi-demethylation of genes by blocking the methylation in replicating cells and inhibiting methyltransferases (47). It probably has other effects as well: reversal of partly committed cells to a multipotent state, thereby modifying cellular fate. 5-azacytidine is also reported to promote the maturation of neurons generated from neural or bone marrow derived stem cells (48, 49).

The second step of our new protocol was a robust, combined activation of the PKC and PKA pathways in order to activate pathways redirecting the cells to a neuronal fate. It was first shown long ago that simultaneous activation of the cAMP and PKC signaling pathways is required for neuronal and glial differentiation (46, 50). Previous reports (50, 51) demonstrated that embryonic stem cells could follow a neural fate in response to basic fibroblast growth factor (bFGF) along with activators of the PKA and PKC pathways, resulting in a dopaminergic phenotype in a subset of cells. Human bone marrow stromal stem cells (BMSCs) were also shown to differentiate into neural progenitors in response to increased intracellular cAMP levels (52). Furthermore, as mentioned above, simultaneous activation of the PKC and PKA pathways resulted in transient neurodifferentiation of BMSCs (23). PKC is also involved in neural differentiation regulating neurite development (53, 54). Moreover, intracellular cAMP elevating factors induce neuroendocrine differentiation in human prostate carcinoma cells (55), neuronal differentiation in C6 glioma cells (56) and elongation of processes in the MCD-1 medulloblastoma cell line (57).

The final step after the dedifferentiation and induction steps was the use of a mixture of conventional neuronal differentiation factors to promote neurodifferentiation. The importance of neurotrophin-3 (NT-3) and nerve growth factor (NGF) in the induction of advanced neuronal development has already been described (58). bFGF, EGF and retinoic acid were also reported to be key regulators of neural stem cell proliferation and neural commitment (15, 21).

Our three step differentiation procedure resulted in a robust differentiation of both DPSC and PDLSC cultures towards neural lineages. At the end of the differentiation, most cells displayed complex neuronal morphology showing both bipolar and multipolar forms. In both pulp and periodontal derived cultures morphological changes were accompanied by a similar increase in the expression of the neuronal marker NSE, and a sharp decrease in the expression of the mesenchymal marker vimentin. Our immunocytochemical observations corresponded well with the real time RNA expression data. These clearly suggest that both DPSC and PDLSC cultures are capable of massive cell differentiation at least as far as cell morphology, gene expression profile, and molecular marker expression is concerned. In a very recent study parallel with the present work we provided evidence that DPSC cells subjected to our newly developed neurodifferentiation protocol showed functional signs of a neuronal phenotype (59). By using patch clamp technology, we provided evidence for the presence in differentiated DPSC cells of functional voltage gated sodium channels and voltage gated potassium channels (59), two neuron-specific channels. Coexistence of these channels is regarded as a basic criterion for functional neuronal cell identification. It needs further careful investigations whether PDLSC cells are also able to differentiate into cells exhibiting the functional characteristics of true neuronal cells.

Taken together, in the present work we report the isolation, culture and characterization of stem cell cultures of both human dental pulp and periodontal ligament origin. Furthermore, our data clearly show that both DPSC and PDLSC cultures can differentiate into either osteogenic or to a neuronal fate in response to appropriate pharmacological treatments. Further investigations are still necessary to optimize these procedures, enabling the utilization of the differentiation potential of human stem cell cultures of dental origin. Nevertheless, it is already clear that both the human dental pulp and the periodontal ligament is a potential source for tissue engineering not only in aspects related to dental bone regeneration, but also for neuroregenerative applications.

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Author's address: Dr. Gabor Varga, Department of Oral Biology; Semmelweis University, H-1089 Budapest, Nagyvárad tér 4, Hungary; Phone: +36-1-210-4415; Fax: +36-1-210-4421; E-mail: varga-g@net.sote.hu