High-purity Hepatic Lineage Differentiated from Dental Pulp Stem Cells in Serum-free Medium

Nikolay Ishkitiev, DMD, Ken Yaegaki, PhD,* Toshio Imai, PhD,* Tomoko Tanaka, PhD,* Taka Nakahara, PhD,† Hiroshi Ishikawa, PhD,† Vanyo Mitev, PhD,‡ and Markus Haapasalo, PhD§*

Abstract

Introduction: We have previously differentiated hepatocyte like cells from deciduous tooth pulp stem and extracted third molar pulp stem cells with a protocol that used fetal bovine serum, but it showed high contaminations of nondifferentiated cells. Both the lower purity of hepatically differentiated cells and usage of serum are obstacles for application of cell therapy or regenerative medicine. Objective of this study was to investigate the capacity for and purity of hepatocyte-like differentiation of CD117-positive dental pulp stem cells without serum. **Methods:** Mesenchymal cells from human deciduous and extracted third molar pulp were isolated and expanded *in vitro*. We separated CD117-positive cells by using a magnetic-activated cell sorter. The cells were characterized immunofluorescently by using known stem cell markers. For hepatic differentiation, the media were supplemented with hepatic growth factor, insulin-transferrin-selenium-x, dexamethasone, and oncostatin M. Expression of hepatic markers alpha fetoprotein, albumin, hepatic nuclear factor-4 alpha, insulin-like growth factor-1, and carbamoyl phosphate synthetase was examined immunofluorescently after differentiation. The amount of differentiated cells was assessed by using flow cytometry. Glycogen storage and urea concentration in the medium were defined. **Results:** Both cell cultures demonstrated a number of cells positive for all tested hepatic markers after differentiation, ie, albumin-positive cells were almost 90% of differentiated deciduous pulp cells. The concentration of urea in the media increased significantly after differentiation. Significant amount of cytoplasmic glycogen storage was found in the cells. **Conclusions:** Without serum both cell types differentiated into high-purity hepatocyte-like cells. These cells offer a source for hepatocyte lineage differentiation for transplantation in the future. *(J Endod 2012;38:475–480)*

Key Words

Adult stem cells, dental pulp, hepatic differentiation, serum-free medium, stem cell pluripotency

We have recently differentiated a hepatic lineage of cells from human dental pulp cells (1), because dental pulp stem cells (DPSCs) are an easily accessed and convenient reserve of stem cells compared with bone marrow stem cells (BMSCs). In the study we found that the purity of hepatic differentiated cells was not to be high (1). An increase of the undifferentiated cells involving stem cells might be one of the concerns for future cell therapy or regenerative medicine because of the possibility of malignancy (2). Many previous studies have also characterized hepatic differentiation from stem cells by using hepatic marker expressions, sometimes at the mRNA level (3–6). Most studies with mesenchymal cells do not describe the purity of the cell lineage from stem cells. Jin et al (4) reported that 68% of the cells were differentiated into alpha fetoprotein/cytokeratin 18 (CK18) double-positive cells, but the cells were negative to albumin. The purity of cell lineage from the stromal stem cells in previous research might not be high. Moreover, even in lineage from human embryonic stem cells (hESCs), the purity of differentiated albumin-positive cells was only 67% (7), although higher purity was expected because of hESCs.

CD117 (proto-oncogene c-kit or stem cell growth factor receptor) is a surface marker present in stem cells from human exfoliating teeth (SHED) and adult human DPSCs from extracted third molars. CD117 has been found in many nondifferentiated cell types, including dental pulp cells, but it disappears when the cells undergo differentiation (8). We have previously reported that 50%–60% of SHED or DPSCs expressed CD117 (1). In recent years, dramatic improvement in magnetic cell separation techniques has made it more popular for isolating target cells compared with the use of a flow cytometer. Magnetic separation might not affect the integrity and scrupulousness of DNA in nuclei because fluorescent dye is not used (9, 10). This is a huge advantage to use magnetic separation for future regenerative medicine. Moreover, magnetic separation requires fewer cells than other separation methods and therefore can be applied to the small numbers of original cells residing in the dental pulp. Recently, we developed a protocol that uses CD117 antibody and magnetic separation techniques for separating stem cells from human dental pulp (11). Furthermore, the previous studies used a 10% fetal bovine serum (FBS)–complemented medium for the differentiation. The presence of animal products in the medium for differentiation also presents a huge obstacle for application of *in vitro* cell therapy methods in clinical practice. FBS has been advanced as a cause of delayed hypersensitivity reaction and to be a potential vector for prion transmission (12). Even autologous serum might

From the *Department of Oral Health and †Section of Developmental and Regenerative Dentistry, Nippon Dental University, Tokyo, Japan; ‡Department of Chemistry and Biochemistry, Medical University, Sofia, Bulgaria; and §Department of Oral Biological and Medical Sciences, University of British Columbia, Vancouver, British Columbia, Canada.

Address requests for reprints to Dr Ken Yaegaki, Nippon Dental University, Department of Oral Health, 1-9-20 Chiyoda-ku, 102-8159 Tokyo, Japan. E-mail address: yaegaki-k@tky.ndu.ac.jp

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cause unknown latent biological reactions during the differentiation process of stem cells. In regenerative medicine, we must lower the possibility of such an obstruction as much as possible. Therefore, we have recently developed a serum-free medium (SFM) for the differentiation of human DPSCs (13).

In this study we investigated a novel protocol for differentiating SHED and DPSCs into a high-purity hepatic lineage. We assessed the capacity of the CD117-positive cell fraction to proliferate in SFM. Furthermore, we determined the potential of the cultured SHED and DPSCs to differentiate into a hepatogenic lineage by using SFM during the differentiation process.

Materials and Methods

Primary Cultures from Human Tooth Pulp Cells

All protocols for this study were reviewed and approved by the Research Ethics Committee of Nippon Dental University. Deciduous and maxillary extracted third molars were supplied after their extraction in patients who received treatment at several collaborating dental clinics. Root formation was completed in all third molars. Informed consent was obtained from all subjects who voluntarily agreed to participate in this study. The pulp was accessed through the resorbed tooth root canal and extracted by a sterile barbed broach. Extracted third molar surfaces were cleaned and cut around the cementoenamel junction by using sterilized dental fissure burs and then cracked open to reveal the pulp chamber. The pulp tissue was gently separated from the crown and root. The pulp was then digested in a solution of 3 mg/mL collagenase type I (Wako Pure Chemicals, Osaka, Japan) for 1 hour at 37°C. Cell suspensions of deciduous or extracted third molar pulp were seeded into 25-cm^2 flasks (Iwaki, Tokyo, Japan) and cultured in Dulbecco modified Eagle medium (DMEM) (Invitrogen, Eugene, OR) supplemented with 10% FBS (HyClone, Logan, UT), 100 U/mL penicillin (Meiji, Tokyo, Japan), and 100 mg/mL kanamycin (Meiji). They were incubated at 37°C in 5% CO_2.

To obtain sufficient numbers of cells for magnetic separation, cells were grown in SFM for up to 4 passages. However, to obtain cell attachment, DMEM supplemented with 10% FBS was used only for the first day after every passage. On the second day after passage, the medium was replaced with SFM.

Isolation and Magnetic Separation of CD117-positive Cells

CD117 is a membrane receptor for the stem cell factor and is related to mesenchymal, endothelial, and endodermal lineage cells (8). The cells were incubated with mouse monoclonal human CD117 fluorescein isothiocyanate–conjugated antibody (Invitrogen). After excess antibodies were removed, the cells were further reacted with mouse anti-human CD117 immunoglobulin G conjugated with magnetic microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany); the cell suspension was then loaded into a column placed in the magnetic field of a mini-MACS Separator (Miltenyi Biotec Inc, Auburn, CA). The unlabeled cells ran through the column, whereas the magnetically labeled cells, representing the CD117-positive cell fraction, were retained in the column. After the column was removed from the magnetic field, the CD117-positive cell fraction was obtained.

Hepatogenic Differentiation

On the day after the separation when the cells attached to the surface, the serum-containing medium was replaced with serum-free DMEM supplemented with 1% insulin-transferrin-selenium-x (ITS-X) (Invitrogen) (13, 14) and 100 μg/mL of embryotrophic factor produced according to the method described by Ishiwata et al (15).

When the cells reached 70% confluence, differentiation agents were added to the SFM. Recombinant human hepatocyte growth factor (HGF) (R&D Systems Inc, Minneapolis, MN) 20 ng/mL was added for 5 days; then a mixture of 10 ng/mL oncostatin M (R&D Systems Inc) and 10 nmol/L dexamethasone (Waco Pure Chemical Industries Ltd, Osaka, Japan) was added for another 15 days. All the media were changed every third day.

Immunofluorescence

Magnetically separated cells were subcultured in 4-chamber slides (5000 cells/slide) (Nalge Nunc International, Naperville, IL) to determine the characteristics of the stem cells. After being fixed with 4% paraformaldehyde, the slides were labeled with the different primary antibodies, followed by Alexa Fluor 568-conjugated secondary antibodies (Invitrogen). The following antibodies produced in mouse were used: anti-CD44H, anti-cytokeratin 19 (R&D Systems); anti-alkaline phosphatase, anti-nestin (AbCam, Cambridge, MA); anti-Nanog (Sigma-Aldrich, St Louis, MO), anti-SPARC (secreted protein acidic and rich in cysteine, osteonectin); anti-p63, anti-STRO-1 (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-CD117 (Miltenyi Biotec, Bergich Gladbach, Germany). Anti-Oct3/4 (R&D Systems) produced in rat was also used. Different sample was stained with different antibody; one sample was stained with one antibody. As a secondary antibody, Alexa Fluor 568-conjugated donkey anti-mouse immunoglobulin G (Invitrogen) was used to detect mouse primary antibodies. The samples were washed 3 times with phosphate buffered saline (PBS) after each antibody layer, and the stained cells were observed under a confocal scanning laser fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany).

To determine whether the cells were differentiated into hepatic cells, the characteristic expression of hepatic phenotype markers was examined at mRNA levels or immunohistochemically (3). In this study, we determined the expression of the markers, and then the number of cells positive to each antibody was determined to identify the concentration of hepatic cells, because a higher concentration of the cells is expected to be required for successful future studies of transplantation. The following specific hepatic markers were used in both stem cells from human exfoliating teeth (SHED) and adult human DPSCs: anti-serum albumin; anti-alpha fetoprotein (αFP) (R&D Systems); anti-insulin-like growth factor I (IGF-I) (Raybiotech, Norcross, GA); anti-hepatic nuclear factor-4 alpha (HNF-4α) (Perseus Proteomics, Tokyo, Japan); and carbamoyl phosphate synthetase (CPS-1) (Santa Cruz Biotechnology) (16). Alexa fluor 568-conjugated antibodies (Invitrogen) were used as secondary antibodies.

Flow Cytometric Analysis

Cells were detached with 0.25% trypsin solution (Invitrogen), followed by incubation for 1 hour at room temperature with anti-albumin, anti-αFP, anti-HNF-4α, anti-IGF-I, and anti-CPS-1 antibodies (all at a concentration of 2 μg/mL). Nonlabeled cells were used as a negative control. The cells were analyzed with Guava EasyCyte flow cytometer (Guava Technologies, Billerica, MA).

Glycogen Storage

To determine glycogen storage capability, we used periodic acid–Schiff (PAS) staining. After 4% formaldehyde fixation, the slides were oxidized in 1% periodic acid for 5 minutes and rinsed in pure water. Samples were then treated with Schiff's reagent (Sigma) for 15 minutes, rinsed in deionized water for 5 minutes, and then assessed under a phase contrast light microscope (Nikon Corp, Tokyo, Japan) for glycogen accumulation.

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