Production of Human Dental Pulp Cells with a Medicinal Manufacturing Approach

Maxime Ducret, DDS,[*†‡] *Hugo Fabre, MS,*[*] *Jean-Christophe Farges, DDS, PhD,*[*†‡] *Olivier Degoul, PhD,*[§] *Gianluigi Atzeni,*[§] *Colin McGuckin, PhD,*[§] *Nico Forraz, PhD,*[§] *Frédéric Mallein-Gerin, PhD,*[*] *and Emeline Perrier-Groult, PhD*[*]

Abstract

Introduction: Human dental pulp cells (HDPCs) are generally isolated and cultured with xenogeneic products and in stress conditions that may alter their biological features. However, guidelines from the American Food and Drug Administration and the European Medicines Agency currently recommend the use of protocols compliant with medicinal manufacturing. Our aim was to design an *ex vivo* procedure to produce large amounts of HDPCs for dentin/pulp and bone engineering according to these international recommendations. **Methods:** HDPC isolation was performed from pulp explant cultures. After appropriate serum-free medium selection, cultured HDPCs were immunophenotyped with flow cytometry. Samples were then cryopreserved for 510 days. The post-thaw cell doubling time was determined up to passage 4 (P4). Karyotyping was performed by G-band analysis. Osteo/odontoblastic differentiation capability was determined after culture in a differentiation medium by gene expression analysis of osteo/odontoblast markers and mineralization quantification. **Results:** Immunophenotyping of cultured HDPCs revealed a mesenchymal profile of the cells, some of which also expressed the stem/progenitor cell markers CD271, Stro-1, CD146, or MSCA-1. The post-thaw cell doubling times were stable and similar to fresh HDPCs. Cells displayed no karyotype abnormality. Alkaline phosphatase, osteocalcin, and dentin sialophosphoprotein gene expression and culture mineralization were increased in post-thaw HDPC cultures performed in differentiation medium compared with cultures in control medium. **Conclusions:** We successfully isolated, cryopreserved, and amplified human dental pulp cells with a medicinal manufacturing approach. These findings may constitute a basis on which to investigate how HDPC production can be optimized for human pulp/dentin and bone tissue engineering. *(J Endod 2015;41:1492–1499)*

Key Words

Cryopreservation, human dental pulp, immunophenotyping, MSCA-1, osteo/odontoblast differentiation, tissue engineering

Dental research currently explores the potential of cell-based products and tissue engineering protocols to be used as alternatives to usual pulp/dentin and bone therapies. In this context, stem/progenitor cells appear to be particularly appropriate because of their high expansion ability and differentiation potential both *in vitro* and *in vivo* (1). If bone marrow and adipose tissue are considered potential sources of stem/progenitor cells, painful collection protocols, the decline of the amount of stem/progenitor cells with age, the necessity of general anesthesia, reduced proliferation capacity, and risk of morbidity at the collection site encourage the search for alternative candidates (1, 2). Human impacted third molars are frequently removed for therapeutic reasons and the loose connective tissue they contain; the dental pulp appears to be a valuable source of stem/progenitor cells for pulp/dentin and bone engineering. Indeed, it contains various cell populations that exhibit osteo/odontoblastic differentiation capabilities and that can be cryopreserved for periods of time greater than 6 months (3–5). Interestingly, human dental pulp cell (HDPC) populations were recently successfully used for regenerating human pulp/dentin and bone (6, 7).

Cell-based products for tissue engineering are now referred to as human cellular tissue-based products or advanced therapy medicinal products, and guidelines from the American Code of Federal Regulation of the Food and Drug Administration (21 CFR Part 1271) and the European Medicines Agency (European Directive 1394/2007) define requirements for appropriate cell production. These "good manufacturing practices" include recommendations regarding laboratory cell culture procedures to ensure optimal reproducibility, efficacy, and safety of the final medicinal product (8, 9). In particular, the Food and Drug Administration divides *ex vivo* cultured cells into "minimally" or "more than minimally" manipulated samples according to function of the use or not of procedures "that might alter the biological features of the cells." In this context, most if not all HDPC culture protocols that have been reported so far are unsatisfactory. Indeed, the use of xeno- or allogeneic cell culture media and long-term cell amplification are known to alter the quality of the final cell-based product (10–12). These findings make the design of new HDPC isolation, characterization, cryopreservation, and amplification *ex vivo* procedures that are compliant with good manufacturing practices regarding medicinal products necessary (5, 7, 13).

From the *Laboratoire de Biologie Tissulaire et Ingénierie Thérapeutique, UMR5305 CNRS/Université Lyon 1, Lyon, France; †Université de Lyon, Université Lyon 1, Faculté d'Odontologie, Lyon, France; ‡Hospices Civils de Lyon, Service de Consultations et Traitements Dentaires, Lyon, France; and §CTIBIOTECH, Cell Therapy Research Institute, Meyzieu, France.

Address requests for reprints to Prof Jean-Christophe Farges, Laboratoire de Biologie Tissulaire et Ingénierie Thérapeutique, UMR5305 CNRS/Université Lyon 1, Institut de Biologie et Chimie des Protéines, 7 Passage du Vercors, 69367 Lyon Cedex 07, France. E-mail address: jean-christophe.farges@univ-lyon1.fr

0099-2399/$ - see front matter

http://dx.doi.org/10.1016/j.joen.2015.05.017

The aim of this study was to define a protocol for obtaining a clinical scale number of HDPCs that possess osteo/odontoblastic differentiation potential. Therefore, HDPCs were grown from dental pulp explants on extracellular matrix–coated dishes, and their storage and amplification were performed in serum-free medium (SFM) by using xenogeneic-free products. After appropriate SFM selection, cell immunophenotype, viability, growth kinetics, karyotyping, and differentiation capacity were analyzed to validate our *ex vivo* protocol.

Materials and Methods

Isolation and Amplification of HDPCs

Healthy impacted human third molars were collected from donors aged 13–17 years with informed consent of the patients and their parents in accordance with the World Medical Association's Declaration of Helsinki and following a protocol approved by the local ethics committee. Teeth between Nolla developmental stages 5 (crown almost completed) and 7 (one third root completed) were used (14). Dental pulps were aseptically, gently extirpated from pulp cavities with fine tweezers, and the apical part of the radicular pulp was removed with a scalpel to prevent contamination by dental papilla cells. Pulps were then washed twice with phosphate-buffered saline containing 100 IU/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Saint Aubin, France), referred to as P/S from herein, placed onto a sterile glass slide and cut with a scalpel into 0.5- to 2-mm^3 explants. The latter were cultured on dishes precoated with an equal mixture of human placental collagens I and III at a final concentration of 0.5 μg/cm^2 (ABCellBio, Paris, France). HDPCs outgrowing from the explants (referred to as passage 0[P0]-HDPCs) were detached from the culture dish after 2 weeks of culture with xeno-free recombinant protease TrypLe Select 1X (Life Technologies), counted, and either cryopreserved (see below) or plated (5×10^3 cells/cm^2) for amplification. The same protease was used for all subsequent passages.

Isolation Success Rate, Cell Outgrowth Surface Area, and Metabolic Assays

Three SFMs called SFM-1, SFM-2, and SFM-3 were tested for assessing HDPC outgrowth formation from the explants. Ninety pulp explants from 5 donors were pooled together to reduce interpatient variability and then separated into 3 equal groups to be cultured in SFM-1, SFM-2, or SFM-3. Explants were then seeded on collagen precoated 12-well plates and cultured in SFM-1 (composition: SPE-IV/EBM [a medium containing clinical grade human albumin, α-minimum essential medium (α-MEM), 25 ng/mL rhIGF-1, and 0.33 ng/mL rhFGF-2; ABCellBio] and P/S), SFM-2 (Dulbecco's Modified Eagle's Medium [DMEM]/F-12/Glutamax [Life Technologies], P/S, 5 ng/mL FGF-2 [R&D Systems, Lille, France] and 5 μg/mL insulin [Umuline, Lily, Neuilly-sur-Seine, France]) or SFM-3 (DMEM/F-12/Glutamax, 1% (v/w) Insulin/Transferrin/Selenium [Life Technologies], and P/S). Cultures were performed for 10 days and then fixed with 10% formalin and stained with a 0.5% crystal violet solution. The isolation success rate was defined as the percentage of explants that give rise to at least 1 HDPC outgrowth. The cell-covered surface area around each explant was calculated by using Image J software (National Institutes of Health, Bethesda, MD). For metabolic activity assessment, first-passage (P1) HDPCs from 5 different donors were pooled and then seeded (5×10^3/well) in collagen precoated 96-well plates. Cells were cultured for 68 hours in 200 μL SFM-1, SFM-2, or SFM-3, and then 40 μL of a 5-mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (Sigma-Aldrich, Saint-Quentin Fallavier, France) was added for 4 hours. Formazan product was solubilized with 99% dimethyl sulfoxide (Sigma-Aldrich) for 15 minutes, and metabolic activity was measured as absorbance at 590 nm with a Multiskan FC microplate reader (Thermo Fisher Scientific, Courtaboeuf, France).

Multicolor Flow Cytometry

Three million P1-HDPCs cultured in SFM-1 were stained with 17 fluorochrome-conjugated antibodies (Table 1). The nucleic acid dye 7-Amino-actinomycin D (7AAD; BD Biosciences, Le Pont de Claix, France) was used for the exclusion of nonviable cells. Samples were acquired on a BD FACSCanto II Flow cytometer (BD Biosciences) as uncompensated events and recorded as FCS 3.0 files. Analysis and compensation were performed using FlowJo vX software (FlowJo, Ashland, OR). The percentage of cells positively stained corresponded to the percentage of cells present within a gate established so that <1% of the measured positive events represented nonspecific binding by the fluorochrome-conjugated isotype-matched control. Additional fluorescence minus one controls were used for CD271, Stro-1, CD146, and MSCA-1.

Cryopreservation

HDPCs outgrowing from explants (P0-HDPCs) were detached with TrypLe Select 1X and counted. Cells were suspended (10^6/mL) in a solution containing 10% dimethyl sulfoxide and 90% cryogenic SFM (CRYO3; Stem Alpha, Saint-Genis l'Argentière, France). Cells were thoroughly mixed in cryotubes and immediately transferred to an isopropanol-filled Cryobox (Nalgene, Rochester, NY). Samples were then frozen in liquid nitrogen for 510 days.

Cell Viability, Cumulative Doubling Number, and Doubling Time Determination

Post-thaw P1-HDPC viability was compared with that of fresh P1-HDPCs. Cells were stained with trypan blue to discriminate between live and dead cells, and they were counted with a Cellometer auto T4 (Nexcelom Bioscience, Lawrence, MA). For cumulative doubling number (CDN) and doubling time (DT) analysis, HDPCs from P1 to P4 were plated (5×10^3 cells/cm^2) on T12.5 flasks (Corning Inc, Corning, NY) until they reached 80%–90% confluence. CDN and DT were calculated from the addition of doubling number counts and the time of culture according to the following formulae:

$$\mathrm{CDN} = \ln(\mathrm{nf}/\mathrm{ni})/\ln 2 \quad (1)$$

$$\mathrm{DT} = \mathrm{CT}/\text{doubling number}(\mathrm{nf} = \text{final number of cells at } 80\% \text{ confluence, } \mathrm{ni} = \text{initial number of cells, and } \mathrm{CT} = \text{culture time}) \quad (2)$$

Karyotyping

Post-thaw P4-HDPCs were exposed for 3 hours to 0.7% colcemid (Life Technologies) diluted in the culture medium, and then cells were detached and centrifuged. The pellet was then resuspended in 0.075 mol/L KCl for 2 minutes at room temperature. Cells were centrifuged again, resuspended in methanol acetic acid (3:1) fixative, and stored at −20°C for at least 2 days. G-band staining was performed with the Leishman-Giemsa cocktail.

Osteo/Odontoblastic Differentiation and Mineralization Quantification

Post-thaw HDPCs were plated (4.2×10^3 cells/cm^2) on plastic dishes and amplified. Once confluence was reached (3–4 days), cells