Influence of 2 Cryopreservation Methods to Induce CCL-13 from Dental Pulp Cells

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Abstract

Introduction: Cryopreservation preserves periodontal ligament cells but has a lower success rate with dental pulp cells (DPCs) because it causes inflammation. There are 2 well-known cryopreservation methods that reduce inflammation, slow freezing and rapid freezing, but the effects of the 2 methods on inflammation are not well-established. The purpose of this study was to compare the effects of the 2 different cryopreservation methods on CCL-13 induction from DPCs by using microarrays, real-time polymerase chain reaction (PCR), Western blotting, enzyme-linked immunosorbent assay, and confocal laser scanning microscopy (CLSM). **Methods:** In this study, the concentration of cryoprotectant was fixed, and the methods compared differed with respect to freezing speed. Initially we screened the DPCs of cryopreserved teeth with expression microarrays, and CCL-13 was identified as a differentially expressed gene involved in generalized inflammation. We then compared the expression of CCL-13 after exposing teeth to the 2 cryopreservation methods by using real-time PCR, Western blot, enzyme-linked immunosorbent assay, and CLSM. **Results:** Expression of CCL-13 was up-regulated significantly only in the rapid freezing group, except in measurements made by real-time PCR. CLSM analysis also confirmed this up-regulation visually. **Conclusions:** Rapid freezing increased the expression of CCL-13 in DPCs compared with slow freezing. Understanding the inflammatory effect of cryopreservation should help to establish an optimal cryoprofile to minimize inflammation of DPCs and reduce the need for endodontic treatment. *(J Endod 2013;39:1562–1566)*

Key Words

Autotransplantation, CCL-13, cryopreservation, dental pulp cell, inflammation

Autotransplantation refers to the reposition of embedded, impacted, or erupted autogenous teeth from one site to another in the same individual onto extracted tooth sockets or surgically prepared recipient sites (1–4). The crucial factor affecting the success of the procedure is the time that the extracted teeth stay outside the tooth socket or preservation solution (5, 6). However, immediate autotransplantation may not be possible in every case because the extraction/autotransplantation is not always coincident; therefore, teeth may be cryopreserved to maintain the viability of the cells on the root surface (2). Slow freezing is a stepwise freezing method, with programmed temperature lowering to reduce ice crystal formation and osmotic damage to cells (7), whereas rapid freezing, which is also called vitrification, uses a high-density cryoprotectant medium. Rapid freezing reduces the time of exposure of cells to the toxicity of the cryoprotectant (8), is expensive (9), and may be an effective alternative to slow freezing (10).

Because dental pulp is surrounded by hard tissues, the cryoprotectant cannot easily reach it, so that maintaining the viability of the dental pulp cells (DPCs) during cryopreservation is more difficult than in the case of periodontal ligament cells (11). It has been recommended that dental pulp tissue be removed before/after cryopreservation, followed by autotransplantation (12); therefore, the conventional endodontic treatments or retrograde filling of the root canals should be performed before the implant of the stored teeth in the liquid nitrogen to prevent the failure of the autotransplantation because of the inflamed pulp tissues after the cryopreservation.

Fibroblasts are the most dominant ones in the composition of DPCs (13). Fibroblasts are able to release substances such as cytokines, chemokines, and inflammatory mediators, so it is considered an important connective tissue cell for tissue integrity and repair (14–17). Monocyte chemoattractant protein-4 (MCP-4, formally named CC motif chemokine ligand 13 [CCL-13]) directs the migration of monocytes/macrophages, T lymphocytes, and eosinophils (18). Expression of MCP including CCL-13 by fibroblasts often increases on treatment with proinflammatory cytokines, and their expression pattern suggests involvement in many human diseases associated with

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leukocyte recruitment into sites of inflammation or injury (19). CCL-13 also plays an important role in the initial step of angiogenesis through its recruitment of vascular endothelial cells and macrophages into the inflamed tissue (20).

The purpose of this study was to compare the effects of the 2 different cryopreservation methods on CCL-13 induction from DPCs by using microarrays, real-time polymerase chain reaction (PCR), Western blotting, enzyme-linked immunosorbent assay (ELISA), and confocal laser scanning microscopy (CLSM).

Materials and Methods

Tooth Collection

Intact caries-free, freshly extracted premolars (n = 9) were collected from 3 patients for microarray assay analysis, and 9 healthy premolars were collected from 3 more patients for the other procedures (real-time PCR and ELISA) in the Department of Orthodontics, Kyung Hee Dental Hospital at Gangdong as described previously (21, 22). Material associated with microarray data of this article can be viewed here (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE45502). Tooth collection from human subjects followed a protocol approved by the Kyung Hee Institutional Review Board, and informed written consent was obtained from all patients. Immediately after extraction, the teeth were stored in phosphate-buffered saline (PBS) and transferred to the laboratory. One premolar from each patient was used as control, and the other premolars from the same patient were cryopreserved.

Cryopreservation of Teeth and Collection of DPCs

Once the extracted teeth were collected, they were assigned to the immediately after extraction group and cryopreserved group, and those in the cryopreserved group were divided into rapid freezing and slow freezing groups. Each group consisted of 3 teeth collected from 3 different individuals.

Control Group (Immediately after Extraction). Immediately after the teeth were extracted, dental pulp tissue was collected 1 mm under the cementoenamel junction of vertically cut teeth. Then DPCs were isolated and cultured by the outgrowth method as described previously (22).

Cryopreserved Groups.

Slow Freezing Group. For slow freezing, premolars were placed in cryopreservation vials containing 10% dimethyl sulfoxide (Me_2SO; Sigma Chemical, St Louis, MO) within 5 minutes. For cooling at a constant rate, they were placed in a cell freezer container with 100% isopropanol and stored in −80°C deep freezer for 24 hours; they were then moved to liquid nitrogen at −196°C for 1 week, followed by rapid thawing in a water bath at 37°C for 3 minutes. DPCs were cultured in the same way as in the control group.

Rapid Freezing Group. For rapid freezing, premolars were placed in cryopreservation vials containing 10% dimethyl sulfoxide (Me_2SO; Sigma Chemical) within 5 minutes and immediately placed in liquid nitrogen at −196°C for 1 week. Thawing and DPC culture were as in the slow freezing group.

Gene Expression Analysis by Using Microarrays

One-microgram samples of total RNA from the 3 sets of culture DPCs were mixed with T7 promoter primer mix and incubated at 65°C for 10 minutes. The cDNA master mix (5X first strand buffer, 0.1 mol/L dithiothreitol [DTT], 10 mmol/L deoxyribonucleoside triphosphate [dNTP] mix, RNase-Out, and MMLV-RT) was prepared and added to the reaction mix. The samples were incubated at 40°C for 2 hours and then at 65°C for 15 minutes to complete synthesis of the cDNA. Transcription master mix was prepared according to manufacturer's instructions (4X transcription buffer, 0.1 mol/L DTT, dNTP mix, 50% polyethylene glycol, RNase-Out, inorganic pyrophosphatase, T7-RNA polymerase, and cyanine 3-CTP). Transcription of cDNA was performed by adding the transcription master mix to the cDNA reaction samples and incubating at 40°C for 2 hours. Amplified and labeled cRNA was purified with cRNA Cleanup Module (Agilent Technology, Santa Clara, CA) and quantified with an ND-1000 spectrophotometer (NanoDrop Technologies, Inc, Wilmington, DE). After checking the labeling efficiency, the cRNA was fragmented by adding 10× blocking agent and 25× fragmentation buffer and incubating at 60°C for 30 minutes. The fragmented cRNA was resuspended in 2× hybridization buffer and directly pipetted into an assembled Agilent Human Oligo Microarray (44K). The array was hybridized at 65°C for 17 hours in an Agilent Hybridization oven and washed following the manufacturer's instructions. Hybridized bands were scanned and quantified with an Agilent DNA microarray scanner. Expression level of genes was obtained from the ratio between control and rapid or slow freezing DPC fluorescent signal intensity. The ratios were calculated by independent analyses of each couple of samples (rapid freezing-I/control-I and slow freezing-I/control-I, etc); then, the average of ratios obtained was used for fold change analysis. Normalization and identification of genes with at least 2-fold differences between control and cryopreserved specimens were performed by using Agilent Feature Extraction Software.

Quantitative Real-time PCR

Total RNA from each group of DPCs was extracted by using Trizol. The RNA concentration was measured with a NanoDrop 1000 instrument. Samples containing 1 μL RNA were reverse transcribed with an iScriptcDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instruction. The cDNA was stored in −20°C. The pattern of CCL-13 transcripts was examined by quantitative real-time PCR analysis by using TaqMan Gene Expression Assays (Applied Biosystems Inc, Foster City, CA) and a predesigned probe and primer (gene ID: CCL-13, Hs00234646_m1; glyceraldehyde-3-phosphate dehydrogenase, Hs99999905_m1). Expression was quantified by Chromo4 Reverse Transcription-Polymerase Chain Reaction analysis (Bio-Rad Laboratories, Hemel Hempstead, UK) and IQ Supermix (Bio-Rad Laboratories, Hercules, CA). To obtain relative levels of gene expression, we used MJ Opticon Monitor Analysis Software (Bio-Rad Laboratories, Hemel Hempstead, UK). All experiments were performed in triplicate.

ELISAs

CCL-13 was measured with a human CCL-13 ELISA kit (Abnova, Walnut, CA) according to the manufacturer's instructions. All experiments were performed in triplicate.

Western Blot Analyses

Cells were washed with a PBS solution and dissolved in cold RIPA buffer (Cell Signaling, Danvers, MA). Supernatants were collected after incubation for 24 hours in serum-free culture medium and concentrated in a Centricon-YM 10 filter device (Millipore, Schwalbach, Germany). Protein concentration was quantified with a protein assay kit (Bio-Rad Laboratories, Hercules, CA). Cell lysates and concentrated supernatants were separated by 12% sodium dodecylsulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride transfer membrane (Amersham Phamacia Biotec, Buckinghamshire, UK). The primary antibody used was anti-human MCP-4 polyclonal antibody diluted 1:1000 (Antibodies-online, Atlanta, GA). Secondary