Laser capture microdissection from formaldehyde-fixated and demineralized paraffin embedded tissues

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Laser capture microdissection (LCM) allows for the microscopic procurement of specific cell types from tissue sections that can then be used for gene expression analyses. In conventional LCM, frozen tissues are normally used for molecular analyses. Some recent studies in cancer research demonstrated that it is possible to carry out gene expression analyses of formaldehyde-fixated paraffin embedded (FFPE) material. However, it is still unclear whether LCM from FFPE tissue sections that had been demineralized allows performing quantitative gene expression analyses. Thus, we performed real-time PCR analysis of specific genes such as dentin sialophosphoprotein (DSPP) in odontoblasts microdissected from engineered dental pulp-like tissues that had been fixed with formaldehyde for 24 hours, demineralized with 10% formic acid for 5-7 days, and embedded in paraffin. The expression of dentin sialophosphoprotein and 18S mRNA was detected and quantified. This method allows for quantitative gene expression analyses in FFPE tissue samples that had been demineralized.

Keywords: laser capture microdissection, tissue engineering, dental pulp, real-time PCR, dentin sialophosphoprotein

1. Introduction

Laser capture microdissection (LCM) allows microscopic procurement of specific cell types from tissue sections that can then be used for gene expression analysis [1]. To date, formaldehyde as a 10% neutral buffered formalin is the most widely used fixative for various types of tissues. As with DNA, formaldehyde reacts with RNA forming an N-methylol (N-CH₂OH) followed by an electrophilic attack to form a methylene bridge. Adenine is the most susceptible nucleotide to the electrophilic attack and it is likely that the adenosine within the mRNA sequence and the poly(A) tail of mRNA are modified in the formaldehyde-fixated paraffin embedded (FFPE) tissue sections to varying degrees. Thus, it is normally considered that RNA isolated from FFPE sections is less suitable for reverse transcription (cDNA synthesis), than RNA isolated from frozen tissue sections [2]. However, gene expression analysis from microdissected FFPE tissues has been successfully conducted in some papers [3-5]. We have recently reported a method for LCM analysis of gene expression from Factor VIII immunostained endothelial cells in the FFPE tissues [4,5]. This method allows retrieving mRNA from a pre-determined cell type in histologically defined areas. However, it is still unclear whether LCM from FFPE tissue sections that had been demineralized allows for quantitative gene expression analysis.

Recently, we have also developed a method of engineering dental pulp-like tissues using stem cells from exfoliated deciduous teeth (SHED) co-implanted with endothelial cells [6]. Thus, in this analyasis, we performed real-time polymerase chain reaction (PCR) for dentin sialophosphoprotein (DSPP) in odontoblasts cells microdissected from the engineered dental pulp-like tissues that had been demineralized and embedded in paraffin.

2. Tooth slice and scaffold preparation

Freshly extracted third molars (n=30) were collected with informed consent and institutional review board approval. Residual soft tissues were removed with a scalpel, and the dental surfaces were wiped down with 70% ethanol. The teeth were then transversally cut at the cervical region with a diamond-edged blade at low speed under cooling with sterile phosphate-buffered saline (PBS, Mediatech, Inc) to obtain slices of approximately 1 mm thickness. Pulp tissue was removed with forceps without touching the dentinal walls to prevent the removal of the predentin layer. The pulp cavity of each tooth slice was filled with sieved sodium chloride (250–450 µm) and poly-L-lactic acid (PLLA) (molecular weight, 250,000 g/mol; Boehringer Ingelheim, Germany) dissolved with chloroform [7]. After polymerization of PLLA, the salt that was used as porogen was completely removed with distilled water. The day before transplantation into murine hosts, the tooth slice/scaffolds were hydrophilized in 100% ethanol for 5 minutes.
90% ethanol for 5 minutes, 80% ethanol for 5 minutes, 70% ethanol for 5 minutes, and in sterile PBS for 8 hours at 4°C.

3. Engineering of dental pulp-like tissues

Stem cells from human exfoliated deciduous teeth (SHED) [8] were cultured in alpha modification of Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 20% fetal calf serum (Equitech-Bio, Kerrville, TX), 100 mol/L l-ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, MO), 2 mmol/L l-glutamine, 100 units/mL penicillin, 100 g/mL streptomycin (Invitrogen) at 37 °C in 5% CO2. Human dermal microvessel endothelial cells (HDMEC; Cambrex, Walkersville, MD, USA) were cultured in EGM2-MV (Cambrex).

Tooth slice/scaffolds were divided into 2 groups: (1) 0.8 × 10⁵ SHED + 7.2 x 10⁵ HDMEC cells; or (2) control, empty scaffolds (no cells). Cells were resuspended in a 1:1 mixture of growth factor reduced Matrigel (BD Biosciences, Bedford, MA) and cell culture medium (total volume of 18 µL per scaffold) to allow for their seeding in the scaffolds. The tooth slice/scaffolds containing cells were incubated for 30 minutes at 37°C to allow for the setting of the Matrigel [7]. Control scaffolds received 36 µL of a 1:1 mixture of Matrigel and cell culture medium without cells. The tooth slice/scaffolds were implanted bilaterally in the subcutaneous tissue of the dorsum of 5- to 7-week-old male immunodeficient mice (CB.17 SCID; Charles River, Wilmington, MA). After 14 or 21 days, the implants were retrieved. In addition, tooth slices were prepared from healthy third molars (n=3), but their pulps were not removed [9]. These tooth slices served as additional controls for our experiments.

4. Tissue preparation

Tissue fixation and demineralization was performed as follows: fixation of the tissues with 10% neutral buffered formaldehyde for 24 hours at 4°C, and demineralization with 10% formic acid for 5-7 days at 4°C. Paraffin embedding was performed as follows: dehydration in 70% ethanol for 30 minutes, 90% ethanol for 1 hour, 95% ethanol for 30 minutes at 4°C, and 3 times in 100% ethanol for 1 hour at room temperature, immersion 2 times in xylene for 1 hour at room temperature, 4 times in infiltrating paraffin for 30 minutes at 58°C, and embedding in paraffin.

5. Sectioning and staining

Sectioning of the paraffin embedded samples at 5-µm thickness was performed on a microtome with a new sterile disposable blade. Sample sections were mounted on poly-L-lysine coated glass foil covered polyethylene naphthalate (PEN) slides for LCM (Leica Microsystems, Bannock Burn, IL). The slides were dried in an incubator at 35 °C for 6 hours. Nuclear staining by hematoxylin was performed as follows: deparaffination of the slides twice in xylene for 3 min at room temperature, washing 3 times in 100% ethanol for 30 seconds, 90% ethanol for 30 sec, 70% ethanol for 1 minute, and in RNase-free water for 30 sec at 4°C, immersion in Gill No.3’ hematoxylin (Sigma-Aldrich, Deisenhonen, Germany) for 5-10 seconds at room temperature, followed by washing with RNase-free water for 30 sec at 4°C. The slides were dried for 1-3 hours at 4°C.

6. LCM

We used the Leica AS LMD system that uses a pulsed 337 nm ultraviolet laser on an upright microscope. The laser beam can be moved with a software-controlled mirror system that allows selecting target cells and tissues. Target cells can be preselected on a monitor with a freehand drawing tool, and then the computer-controlled mirror moves the laser beam along the pre-selected path and the target cells are excised from the section. The dissected part then falls into a PCR tube under gravity. The collection by gravity ensures quick and contamination-free processing of the dissected tissue sections. The thickness and width of the cutting line can be controlled for each tissue.

In this analysis, a two-step dissection strategy was performed. First, tissues of the odontoblast layer just underneath the dentin were dissected into individual tubes filled with a storing medium (RNAlater®, Ambion, Austin, TX), and placed immediately on ice. Second, tissues underneath the odontoblast layer were dissected into other individual tubes filled with RNAlater®, and placed immediately on ice (Fig. 1). Control groups of empty scaffolds without cells or tooth slices from healthy human third molars were dissected by the same way as mentioned above (Fig. 1).
Fig. 1 Step-by-step characterization of the technique based on LCM used for retrieval of either the odontoblastic layer or the tissue underneath the odontoblastic layer from FFPE tissue sections that had been demineralized. (a) H&E staining of the engineered dental pulp-like tissue after 21 days of implantation. D: dentin. P: engineered dental pulp. Arrowheads: odontoblastic layer. Bar: 100 µm. (b) Air dried slide of the engineered dental pulp-like tissue. D: dentin. P: engineered dental pulp. (c) Retrieval of the odontoblastic layer. (d) Removal of the tissue underneath the odontoblastic layer.

7. RNA isolation

After LCM, the collected samples were incubated with 50 µL of TRIZOL® Reagent for 5 minutes at room temperature and an equal amount of chloroform was added, shaken for 30-60 seconds and incubated for 5 min on ice. The samples were centrifuged at 13,000 rpm for 15 minutes at 4°C. After centrifugation, the mixture separates into three layers: a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA is contained exclusively in the aqueous phase. Then, the aqueous phase was transferred to a fresh tube. An equal amount of isopropyl alcohol (RNA precipitant) was added to the transferred aqueous phase, incubated at 4°C for 10 minutes, and centrifuged at 13,000 rpm for 15 minutes at 4°C. After-centrifugation, the supernatant was discarded. 70-75 % ethanol in RNase-free water was added to the sample, and centrifuged at 6,000 ~7,500 rpm for 5 minutes at 4°C. The supernatant was discarded again. After drying the sample in a chemical hood, RNase-free water was added to the RNA sample.

8. RNA clean up

RNA clean up was performed by using RNeasy Mini Kit (Qiagen) at room temperature. The sample volume was adjusted to 100µL with RNase-free water, and then it was added 350 µL of Buffer RLT in which 10 µL/mL of β-mercaptoethanol had been added. After the sample was briefly mixed, 250 µL of 100 % ethanol was added, and mixed by pipetting without centrifuging. Total sample (700 µL) was transferred to an RNeasy Mini spin column placed in a 2 ml supplied collection tube and centrifuged for 15 seconds at 8,000 rpm. The flow-through was discarded. Then, 350 µL of Buffer RW1 was added to the RNeasy spin column, and centrifuged for 15 seconds at 10,000 rpm to wash the spin column membrane and discard the flow-through. After that, 80 µL of DNase I incubation mix (10 µL DNase I stock solution and 70 µL of Buffer RDD, supplied with the RNase-Free DNase Set) was added directly to the RNeasy spin column membrane, and placed at room temperature for 15 min. 350 µL of Buffer RW1 was added to the RNeasy
spin column and centrifuged for 15 seconds at 10,000 rpm. The flow-through was again discarded. Then, 500 µL of Buffer RPE was added to the RNeasy spin column, and centrifuged for 15 seconds at 10,000 rpm and the flow-through was discarded. 500 µL of Buffer RPE was added to the RNeasy spin column, and centrifuged for 2 min at 10,000 rpm. The RNeasy spin column was placed in a new 1.5 ml collection tube, and 30–50 µL of RNase-free water was added directly to the spin column membrane, and centrifuged for 1 min at 10,000 rpm to elute the RNA. The purity and quantity of total RNA obtained were calculated with a photometer (BioPhotometer plus. Eppendorf, Wesseling-Berzdorf, Germany). The RNA purity was considered adequate when the OD260/OD280 ratio was indicated from 1.9 to 2.1.

9. cDNA synthesis

Synthesis of cDNA from total RNA samples is the first step in the two-step reverse transcription (RT)-PCR experiment. The TaqMan® Reverse Transcription Reagents (Applied Biosystems) was used to prepare RT reaction mix (final volume 10 µL). A 100 µL RT reaction efficiently converts a maximum of 2 µg total RNA to cDNA. All the nonenzymatic components: 3.85 µL RNase-free water, 1.0 µL 10X RT Buffer, 2.2 µL 25 mM MgCl2, 2.0 µL deoxyNTPs Mixture, and 0.5 µL random hexamers, were prepared and vortexed briefly. Then, 0.2 µL RNase inhibitor, 0.25 µL MultiScribe reverse transcriptase, and the RNA were added and mixed by inverting the tube. The RT reaction volume can vary from 10 µL to 100 µL. Increasing the RT reaction volume will reduce the total number of reactions. Thermal cycling for RT Reactions was performed at 25°C for 10 minutes (incubation), 48 °C for 30 minutes (RT), and 95 °C for 5 minutes (RT inactivation).

10. Real-time PCR for DSPP and 18S

Quantitative gene expression analysis by using Real-time PCR is a reliable approach for combination with LCM. In RNA quantitation assays, the TaqMan® Universal PCR Master Mix (Applied Biosystems) is used in the second step of the two-step RT-PCR protocol. Total RNA at 100 ng/50 µL from PCR reaction mix was used. Probe and primer sets of TaqMan® Gene Expression Assays (Hs00171962_m1; DSPP, and Hs99999901_s1; 18S) were obtained from Applied Biosystems. The PCR reaction mix for one reaction is 2.5 µL of 20X TaqMan® Gene Expression Assays, 25.0 µL of 2X TaqMan® Gene Expression Master Mix, 1.0 µL of cDNA template, and 21.5 µL of RNase-free water.

To compare the mRNA levels between the samples, the standard curve method was used. In this method, the quantity is determined by comparison to a standard curve generated using serial dilutions of a standard RNA. RT-PCR reaction mix (final volume 50 µL for one reaction mix composed by 25.0 µL of 2X Master Mix, 1.25 µL of 40X MultiScribe and RNase Inhibitor Mix, 2.5 µL of 20X TaqMan® Gene Expression Assays (DSPP or 18S), 2.0 µL of the standard RNA, and 19.25 µL of RNase-free water is made by TaqMan® One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems). Both the PCR and RT-PCR reaction mix were transferred into a 96-well clear optical reaction plate. Then, the plate was centrifuged and loaded into an ABI7700 Sequence Detection System (Applied Biosystems). Thus, the data were quantified using the standard curve method and normalized by the data of 18S (Fig.2).
Fig. 2 Real-time PCR used to quantify DSPP mRNA expression in cells from the odontoblastic layer and from tissues underneath the odontoblastic layer retrieved from FFPE tissue sections that had been demineralized. Specimens were collected from engineered dental pulp tissues after 21 days of implantation. Data obtained from real-time PCR experiments reflect the expression level of DSPP normalized by 18S. *, $P < 0.05$ (t test).

11. Conclusion

The method presented here allows for the quantitative analysis of gene expression in paraffin-embedded tissue sections from specimens that have been demineralized. This method constitutes a new approach for gene expression studies of mineralized tissues such as bone and teeth, and opens the door for the acquisition of new data from archived specimens.

References


