Asian Journal of Biochemistry

ISSN 1815-9923

Academic
Journals Inc.

www.academicjournals.com

Asian Journal of Biochemistry 4 (2): 36-44, 2009
ISSN 1815-9923

Molecular Existence of Mature Odontoblast and Osteoblast Cells in Adult Human Pulp Tissues

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Abstract: The dental pulp tissue is essential in dentine development. The existence of Dental Pulp Stem Cells (DPSCs), i.e., osteoblast and odontoblast are to assist in dentine repair and tooth regeneration. The existence of osteoblast that secreted bone matrix directly from pulp tissue has not been reported. The purpose of this study is to determine the existence of odontoblast and osteoblast cells excavated directly from pulp tissues by using molecular markers. The isolated RNA expressing two gene markers, i.e., dentin sialophosphoprotein and osteocalcin which were secreted by odontoblast and osteoblast cells, respectively. The expression of dentin sialophosphoprotein and osteocalcin demonstrated that both odontoblast and osteoblast cells exist in adult human pulp tissues.

Key words: Pulp tissue, osteoblast, odontoblast, osteocalcin, dentin sialophosphoprotein.

INTRODUCTION

Dental pulp tissue located at the centre region of tooth contains soft connective tissues and cells such as odontoblast, fibroblast as well as defence cells. This soft and unmineralized connective tissue provides nutrients and sensory properties to the dentine (Shiba *et al*., 2003). The major structure of pulp consists of several layers of odontoblast, cell free, cell rich and finally loose vascular connective tissue. The large intercellular space in pulp constitutes of type I and type III collagen fibrils (56 and 41%, respectively). The dental pulp also contains cells that are responsible for the formation and turnover of complex non-mineralized extracellular matrices (Goldberg and Smith, 2004). Most of the pulp cells are fibroblast-like cells that produce Complex Extracellular Matrices (CEM), which is substantially different from that of dentin and other soft connective tissues.

Majority of the adult dental pulp contains of macrophages, nerves and capillary cells (Rodd and Boissonade, 2002). In human, the Dental Pulp Cells (DPCs) has also heterogenous population that contains progenitor/stem cells of odontoblast lineage (Alliot-Licht *et al*., 2005; Huang *et al*., 2006). This type of cells has the ability to proliferate and differentiate into odontoblast under conditions such as caries and trauma (Murray *et al*., 2000, 2002). The differentiated odontoblast is believed to come from a subgroup of precursor cells, i.e., neural-crest derived cells which reside in the dental pulp.

Odontoblast is highly specialized cell that aligned in a single layer at the edge of the dental pulp. Therefore, they are the first pulpal cells that are in contact with dental pathogens (Dommisch *et al*., 2005). They play a pivotal role in dental defensive activities during microbial invasion (Jiang *et al*., 2006) and also express β-defensin (Dommisch *et al*., 2006), Transforming Growth Factor (TGF)-β

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(Piattelli *et al*., 2004) that induce antimicrobial and anti-inflammatory activities respectively. Odontoblast cells also produce proinflammatory chemokine interleukin (IL-8) (Huang *et al*., 1999) and secrete several collagenous and non-collagenous proteins to form a unique extracellular matrix. Type I collagen, proteoglycans and dentin sialophosphoprotein (DSPP) are among the few molecules that are synthesized and secreted by the odontoblast (Sreenath *et al*., 2003). Among these molecules, DSPP has been used as a marker for odontoblast differentiation and is known as dentin-specific protein (Feng *et al*., 1998). On the other hand, osteoblast derived from mesenchymal stem cells from various development stages of a primitive single cell type (Aubin and Liu, 1996). They are round cells with an organelle-rich cytoplasm and play an essential role during bone formation. Mature osteoblast expressed the extracellular matrix protein, i.e., osteocalcin (OCN), which associated with the increased of bone matrix mineralization and decreased in osteoblastic cells proliferation (Candeliere *et al*., 2001).

In present study, the determination of DSPP and OCN will prove molecularly the existence of odontoblast and osteoblast cells in purified mRNA from human dental pulp. It is also show that cells consist of the common progenitor of odontoblast and osteoblast cells will be differentiated into specific cells upon existence of specific inducer such as differentiated factors or microenvironments.

MATERIALS AND METHODS

Sample Preparation

Human premolar teeth (18-25 years old) extracted from normal patients were collected from the Department of Orthodontic, Dental Faculty of UKM, Kuala Lumpur. The teeth were grooved vertically from the centre of mesial and dental marginal ridge until cemento enamel junction using dental fissure burs without revealing the pulp chamber. The extracted teeth were immediately frozen into liquid nitrogen. The teeth was cut into two-halves and the dental pulp tissues were removed from the crown and root using dental probe and barbed broach before been homogenized in TriReagent. The homogenized tissues were allowed to stand for 5 min at room temperature to ensure complete dissociation of nucleoprotein complexes. Approximately, 0.2 mL chloroform per mL of TriReagent was added into the samples and centrifuged at 12, 000 g for 15 min at 4°C to separate the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA) and a colorless upper aqueous phase (containing RNA).

RNA Isolation of Human Pulp Tissues

The aqueous phase which contains RNA was transferred to a fresh tube before adding with 0.5 mL of absolute isopropanol per mL of used TriReagent. The mixtures were allowed to stand for 5-10 min at room temperature and centrifuged at 12, 000 g for 10 min at 4°C to form a pellet. The RNA pellet was precipitated with 1 mL of 75% ethanol followed by centrifugation at 7500 g for 5 min at 4°C before air-drying for approximately 5-10 min. An appropriate volume of either formamide, DEPC- treated water or 0.5% SDS solution was added to the pellet and mixed by repeated pipetting before heated at 55-60°C for 10-15 min. The final preparation of RNA should be free of DNA and proteins, and have an approximate 260/280 ratio of 1.7. The isolated RNA was used in RT-PCR amplification.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Amplification

One microgram (1 μg) of RNA sample was subjected to RT-PCR amplification using Access RT-PCR kit (Promega) in a total volume of 20 μL. The sample was amplified using specific primers (Table 1). The reaction was incubated in a PCR cycler using programmed cycle indicated in Table 2. The resulting products were separated by 1% agarose gel electrophoresis and the gels were stained with ethidium bromide, destained in water and photographed using Alpha Imaging System (Alpha Innotech) under UV light.

Table 1: Primer Sequences Used in RT-PCR

Gene	Sequence (5'-3')	Expected product (bp)
GAPDH[a]		
$S_{386-402}$	CCATGGAGAAGGCTGGG	195
$AS_{580-561}$	CAAAGTTGTCATGGATGACC	
DSPP[b]		
$S_{111-132}$	CTAAAGAAAATGAAGATAATT	293
$AS_{403-382}$	TAGAAAAACTCTTCCCTCCTAC	
OCN[c]		
S_{24-42}	AGCCCTCACACTCCTCGCC	270
$AS_{293-274}$	GCCTCCTGAAAGCCGATGTG	

[a]GAPDH :Glyceraldehydes-3-phosphate dehydrogenase (NM_002046), [b]DSPP: dentin sialophosphoprotein (NM_014208), [c]OCN: osteocalcin (NM_199173), S: sense and AS: anti-sense. Lower case numbering at each sense (S) and anti-sense (AS) primer is nucleotide number of designated gene

Table 2: Reverse transcription and PCR cycling profile

Amplification event	Temperature (°C)	Duration	No. of cycles
1st strand cDNA synthesis	45	45 min	1
AMV RT inactivation and RNA/cDNA/primer denaturation	94	2 min	1
2nd strand cDNA synthesis and PCR amplification			
Denaturation	94	30 sec	
Annealing	55 (GAPDH) 51 (DSPP) 61 (OC)	1 min	40
Extension	68	2 min	
Final extension	68	7 min	1

GAPDH: Glyceraldehydes-3-phosphate dehydrogenase (NM_002046), DSPP: dentin sialophosphoprotein (NM_014208), OCN: Osteocsalcin (NM_199173)

Specific primer based on GenBank nucleotides for human osteocalcin (OCN) was designed using Primer Premier V5 (Table 1). Primer sequences for human dentin sialophosphoprotein (DSPP) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were obtained according to Buchaille *et al*. (2000) and Papagerakis *et al*. (2002), respectively.

Cloning and Sequencing

The amplification products of GAPDH, OCN and DSPP transcripts were gel-eluted using Wizard SV Gel and Purification Clean-Up System (Promega) before cloned into the PCR product cloning vector, i.e., the pGEM-T Easy Vector System (Promega). The vector was then transformed into JM109 competent cells and the plasmids constructed for each gene were identified and confirmed by DNA sequencing using BigDye Terminator sequencing kit (Applied Biosystem). Comparison of cloned human sequence to known sequence (GenBank/EMBL database) was performed using BLAST server at NCBI, which gave percentage of identical nucleotide and protein translations.

RESULTS

RNA Isolation from Human Dental Pulp Tissues

The purified total RNA isolated directly from human pulp tissues were taken from extracted premolar tooth from normal patients. Two bands of RNA, i.e., 28S and 16S of rRNA which were obtained after the electrophoresis (Fig. 1) showed that RNAs were isolated from the pulp tissues.

Presence of OCN and DSPP Transcripts in Human Pulp Tissues

In order to further demonstrate that the RNA was purified from human pulp tissues, positive control amplification was performed using a primer set for glyceraldehydes-3-phosphate dehydrogenase (GAPDH), a house keeping gene (Barber *et al*., 2005). The primer resulted in DNA amplification of 195 bp (Fig. 2a, b; Lane 1). On the other hand, negative controls were performed using each specific primer set with the RNA template that was replaced by sterile water which gave no amplification results (Fig. 2a, b; Lane 3). In order to demonstrate the activation of DSPP and

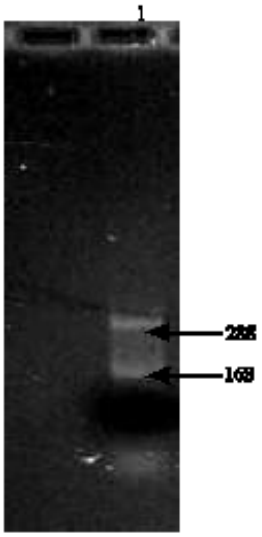

Fig. 1: Two types of rRNA (28S and 16S) were directly obtained from human pulp tissues (lane 1) using TriReagent method and separated by electrophoresis in 1% agarose gel (DEPC treated) followed by staining with ethidium bromide

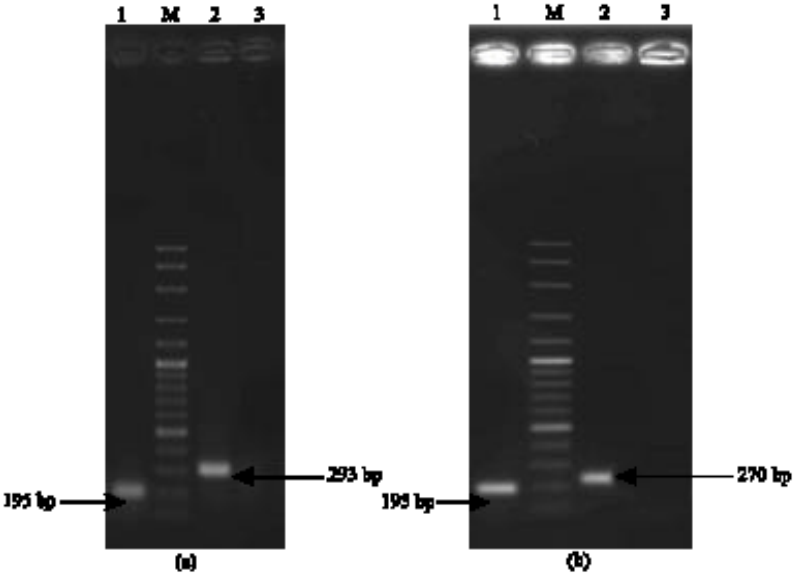

Fig. 2: Activation of DSPP, OCN and GAPDH genes in human dental pulp tissues. RT-PCR was performed on RNAs from pulp tissues with gene specific primers for DSPP, OCN and GAPDH, which was used as a control; (a) Amplification of GAPDH (195 bp, Lane 1) and DSPP (293 bp, Lane 2), (b) Amplification of GAPDH (195 bp, Lane 1) and OCN (270 bp, Lane 2). Negative controls are shown in lane 3 for both (a) and (b). The PCR products were observed by electrophoresis on 1% agarose gel and stained with ethidium bromide. 100 bp DNA ladder was used as marker (M)

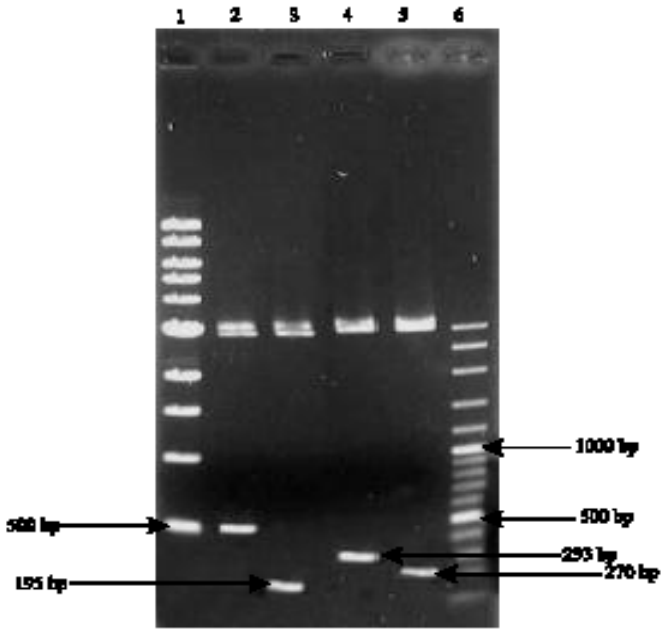

Fig. 3: Digestion of plasmid recombinants using *Eco RI*. The digestion products were separated by electrophoresis in a 1% agarose gel and stained with ethidium bromide. Lane 1: 1 kb DNA ladder (Promega), Lane 2: control insert (Promega), Lane 3: GAPDH clone, Lane 4: DSPP clone, lane 5: OCN clone and Lane 6: 100 bp DNA ladder

Table 3: Sequence analysis results of OCN, DSPP and GAPDH clones using BLASTN program

Genes [Accession No.]	Identities (%)	E-value
Homo sapiens dentin sialophosphoprotein (DSPP) [NM_014208]	100	2e-114
Homo sapiens glyceraldehydes-3-phosphate dehydrogenase (GAPDH) [NM_002046]	99	8e-96
Homo sapiens bone gamma-carboxyglutamate (gla) protein (BGLAP) or Osteocalcin [NM_199173]	89	1e-65

OCN genes in human pulp tissues, the presence of their mRNAs were assessed by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification. DSPP and OCN specific primer sets generated the predicted amplification products of 293 (Fig. 2a; Lane 2) and 270 bp (Fig. 2b; Lane 2) respectively. The amplification products were cloned into pGEM-T vector to confirm that the amplicons were the desired products. Those plasmids which carried the desired genes were digested with a restriction enzyme, i.e., *Eco RI* and resulted in DNA inserts of 195, 293 and 270 bp, respectively (Fig. 3; Lane 3, 4 and 5).

Sequencing Analysis

The amplification products of 270, 195 and 293 bp were successfully cloned into pGEM-T vector. These clones that carry the desired genes for this study were further analyzed by sequencing analysis using universal primers, i.e., T7 and SP6 as forward and reverse primers respectively. Table 3 shows the sequence analysis results from the recombinant plasmids of 195, 293 and 270 bp amplicons. The results showed that the clones were highly similar with the known sequences obtained from BLASTN analysis.

DISCUSSION

The aim of this study was to determine molecularly the existence of odontoblast and osteoblast cells in adult human pulp tissues by the expression of dentin sialophosphoprotein (DSPP) and osteocalcin (OCN) genes. Present results showed that DSPP and OCN genes were activated in the mRNA of adult human pulp tissues when amplified using their specific primers. Most of the previous studies involving molecular mRNA amplification were performed using dental pulp cells culture followed by RNAs isolation from the respective culture. Lopez-Cazaux *et al*. (2006) isolated total RNA from human pulp cells culture and assessed the expression of various dentin and bone related transcripts, i.e., osteonectin (ON), dentin sialophosphoprotein (DSPP), parathyroid hormone/parathyroid hormone-related protein-receptor (PTH/PTHrp-R) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). However, in this study the RNA was isolated and purified directly from excavated adult human pulp tissues which were taken from premolar tooth of normal patients. The isolation and purification of intact RNA was done by using the modifications of guanidium thiocyanate method which involves extraction of guanidium thiocyanate homogenate with phenol-chloroform (Chomczynski and Saachi, 1987). The results from Fig. 1 showed that the modified method was significantly able to reduce the DNA contamination in RNA samples. The modification includes incubation of mixture on ice for 5 min and centrifugation for 15 min. RT-PCR amplification was performed to determine the involvement of two potential genes, i.e., DSPP and OCN, which were activated during odontoblast and osteoblast activity, respectively. Amplification of DSPP and OCN by RT-PCR using their specific primer sets indicated that both genes were found in adult human pulp tissues hence suggested the existence of mature odontoblast and osteoblast cells in pulp tissue. In this study, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), a well-known housekeeping gene was used as our positive control. GAPDH which is the most common housekeeping gene was also used as comparisons during gene expression analysis (Barber *et al*., 2005), as it is constitutively expressed at the same level in mammalian cells and tissues.

The stimulation of morphologically and functionally odontoblast and osteoblast cells is crucial in the maintenance of hard tissue specifically in tooth and bone respectively (Rani and MacDougall, 2000). Osteoblast, i.e., morphologically as round cells, is responsible for the formation of bone, by laying down osteoid and mineralizing it. This cell is derived from mesenchymal stem cells and represents various stages of the development of a primitive single cell type (Aubin and Liu, 1996). Meanwhile, odontoblast is the cells responsible for the formation of dentine, the collagen-based mineralized tissue that forms the bulk of teeth. These cells are post-mitotic neural crest-derived cells, which are also originated from mesenchymal stem cells. Odontoblast cells exhibit a tall columnar shape located at the periphery of the dental pulp and establish a continuous single layer with a clear epithelioid appearance (Arana Chavez and Massa, 2004). Osteoblast and odontoblast are two cell types which present in craniofacial complex. They are originated from ectomesenchymal cells and both of them secrete macromolecules that are necessary for the formation of dentine and alveolar bone via the respective specific matrix-mediated mechanism. However, both cells, i.e., osteoblast and odontoblast, synthesizes several common proteins that involve in calcium and phosphate handling as well as producing highly similar extracelullar matrices such as type I collagen and one of a major mineralized matrix protein, i.e., osteonectin.

Dental pulp contains para-axial mesenchyme-derived cells and cranial neural-crest derived cells (Goldberg and Smith, 2004). These mesenchymal cells and/or stem cells have been thought to be responsible for hard tissue formation (Gronthos *et al*., 2000; Miura *et al*., 2003). In *in vitro* cells culture, these type of cells that originated from the dental pulp have the ability to differentiate into

odontoblast and osteoblast (Ruch *et al*., 1995, Papaccio *et al*., 2006) by the assistance of a molecule called Bone Morphogenic Proteins, i.e., BMPs (Nakashima and Akamine, 2005). BMPs promote the differentiation of mesenchymal cells into odontoblast and osteoblast by inducing the expression of Runx2 and bone matrix proteins. In the adult tooth, dental pulp appears to contain progenitors that can be recruited to differentiate into odontoblast-like cells for tooth repair (Priam *et al*., 2005). Papagerakis *et al*. (2002) reported that the differentiated odontoblast will produce DSPP and this protein is not expressed in undifferentiated and primitive pulp cells. This showed that the mature odontoblast is also present in pulp tissues as expected since mature odontoblast is important during the formation of dentine.

The DSPP protein is a member of the SIBLING (small integrin binding ligand N-linked glycoprotein) family, which also includes osteopontin, bone sialoprotein, dentin matrix protein-1 (DMP1) and matrix extracellular phosphoglycoprotein (MEPE) (Yunfeng *et al*., 2005). The gene that encoded DSPP is located on human chromosome 4q21 and contains multiple phosphorylation sites. This protein is highly acidic and has an arginine-glycine-aspartate (RGD) cell attachment domain as it is thought to play an important role in tissue mineralization (Yunfeng *et al*., 2005). DSPP is the initial translational product of DSPP messenger RNA (mRNA) and then cleaved to dentin phosphoprotein (DPP) and dentin sialoprotein (DSP) (Butler *et al*., 2002). DSPP which is expressed by odontoblasts is essential in dentinogenesis (Zhang *et al*., 2001; Sreenath *et al*., 2003) as it is a dentin-specific protein (Qin *et al*., 2002). As a result, DSPP remains to be a significant marker for odontoblast differentiation.

Osteocalcin (OCN) protein comprises about 2% of the total protein in bone (Knepper-Nikolai *et al*., 2002). It is also known as bone Gla protein and is recognized as a marker for bone formation. It is located on chromosome 1 (1q25-q31) and is regulated by 1, 25-dihydroxy vitamin D_3 (Puchacz *et al*., 1989). OCN is a vitamin-K and vitamin-D dependent protein produced by osteoblasts and is the most abundant. This type of protein is the non-collagenous protein that is most widely studied in bone. OCN studies in developing embryos and bone cell models have validated its high specificity as a marker for the mature osteoblastic phenotype. The *osteocalcin* gene is the most thoroughly studied of all bone-specific genes, serving as a model for regulation by 1, 25 dihydroxy-vitamin D_3, glucocorticoids, growth factors and the transcription factors Runx2/Cbfa1 and Osterix (Knepper-Nikolai *et al*., 2002). It is an osteoblast-specific structural gene which only expressed by fully differentiated osteoblast. Present study had revealed that the existence of OCN in adult human pulp tissues which were not treated by any osteoblastic differentiation medium was also expressed. Our results proved that the undifferentiated and primitive cells in pulp tissue also consist of matured and committed osteoblast cells.

In conclusion, we have successfully isolated total RNA directly from adult human pulp tissues by using a modified acid guanidium thiocyanate-phenol-chloroform method. This modified method not only reduces DNA contamination in the RNA samples but also generates strong 293 and 270 bp amplicons in RT-PCR. The successful amplification of DSPP and OCN in this study showed that the existence of odontoblastic and osteoblastic cells in undifferentiated and primitive pulp cells population that is in adult human pulp tissues.

ACKNOWLEDGMENT

The authors would like to thank Ministry of Science, Technology and Innovation for the Fundamental Grant (02-01-02-SF0245), UKM-GUP-BTK-07-15-197, UKM-ST-01-FRGS0020-2006 and UKM-OUP-TKP-18-84/2008.

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