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Analysis of proteoglycan expression in human dental pulp

Ana Carla Raphaelli Nahás-Scocate[a,b,*], Gabrielle Ferrante Alves de Moraes[a], Helena Bonciani Nader[a], Carolina Meloni Vicente[a], Leny Toma[a]

[a] Departamento de Bioquimica da Universidade Federal de São Paulo, Disciplina de Biologia Molecular, Rua 3 de Maio 100, 04044-020, São Paulo, Brazil
[b] Universidade Guarulhos, Praça Tereza Cristina, 229, Guarulhos, SP, 07023-070, Brazil

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ABSTRACT

Proteoglycans are glycosylated proteins which have covalently attached highly anionic glycosaminoglycans. They can be located on the extracellular matrix, cell membrane or intracellular granules. To date, few studies have reported the presence of proteoglycans in human dental pulp.

Objective: The aim of this study was, therefore, to analyze the expression of lumican, versican and glypican proteoglycans in deciduous and permanent human dental pulp by real-time polymerase chain reaction (q-PCR) and immunofluorescence.

Design: Healthy human dental pulps were used: 13 from permanent teeth (group 1) and eight from deciduous teeth (group 2). Versican, lumican and glypican (glypican-1 to 6) gene expressions were quantitatively evaluated by real-time PCR technique, using the expression of the endogenous gene GAPDH as control. Pulp sections were submitted to immunostaining procedure with fluorescence labelling, the tissues being fixed and incubated with well-characterized monoclonal and polyclonal antibodies against proteoglycan epitopes, including anti-versican and anti-lumican. Comparisons among the groups of the quantitative scores for each proteoglycan were analyzed using the *t*-test and ANOVA ($P < 0.05$).

Results: The real-time PCR analysis showed expression of versican and lumican proteoglycans in the two groups, with significant predominance of lumican gene ($P = 0.03$). Considering the glypican genes, glypican-3 was the proteoglycan most significantly expressed in permanent pulps ($P < 0.001$), while glypican-2 was not expressed in this tissue. The immunofluorescence quantification exhibited no significant differences between lumican and versican among the pulps and groups.

Conclusions: The lumican gene was more expressed than versican and glypican-3 was the isoform more expressed in permanent pulp compared to deciduous.

1. Introduction

Dental pulp is a loose unmineralized connective tissue, surrounded by dentin. It provides nutritional and sensory properties to dentin. Among the cells found in pulp are fibroblasts, stem and vascular cells, pericytes, neural cells, histiocytes/macrophages, dendritic cells, lymphocytes and mast cells. Pulp extracellular matrix comprises a variety of proteins and polysaccharides secreted locally, forming a network. Type I and type III collagen comprises 56 and 41%, respectively, in contrast to type I collagen predominantly in dentin. Pulp extracellular matrix scaffold is an emerging tool in tissue engineering for the reconstruction of tissues. In this context, dental pulp represents a rich source of stem cells (Aulino et al., 2015; Paduano, Marrelli, Palmieri, & Tatullo, 2016; Perniconi et al., 2014; Tatullo et al., 2015).

The proteoglycans described so far are decorin, biglycan and versican, besides hyaluronic acid. Matrix macromolecules include fibronectin, tenascin, bone sialoprotein (BSP), osteopontin (OPN), bone morphogenetic proteins (BMPs), matrix metalloproteinases (MMPs), activin and types IA and II receptors for TGF-β (Benedetto, Siqueira, Mascaro, Araujo, & Bonecker, 2013; Goldberg & Smith, 2004). Proteoglycans (PGs) are extracellular matrix and cell-surface associated macromolecules that vary in form and function (Oksala, Haapasalmi, Hakkinen, Uitto, & Larjava, 1997), consisting of a core protein and one or more covalently attached glycosaminoglycan (GAG) chains. GAGs are linear polysaccharides, formed by repeated disaccharide units, consisting of an amino sugar (N-acetylglucosamine, glucosamine that is variously N-substituted, or *N*-acetylgalactosamine) and a uronic acid (glucuronic acid or iduronic acid) or galactose (Kjellen & Lindahl, 1991; Nakao et al., 2015).

The cell surface proteoglycans include syndecan (four members)

* Corresponding author at: Universidade Guarulhos—UNG, Praça Tereza Cristina, 229, Guarulhos, SP, 07023-070, Brazil.
E-mail addresses: carlanahas@yahoo.com.br (A.C.R. Nahás-Scocate), gabrielledemoraes@yahoo.com.br (G.F.A. de Moraes), hbnader.bioq@epm.br (H.B. Nader), carolmv@yahoo.com (C.M. Vicente), ltoma.bioq@epm.br (L. Toma).

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and glypican (six members). The glypican family is a member of heparan sulfate proteoglycans and it is composed of six isoforms in mammals (GPC-1 to GPC-6) (Murakami Masuda et al., 2010). The glypicans are linked to the cell membrane through a glycosylphosphatidylinositol anchor, which is connected to a hydrophobic sequence in C-terminal portion of the core protein (terminal carboxil group). The size of the core protein is around 60 and 70 kDa (Filmus & Capurro, 2008).

Proteoglycans have been implicated in tissue remodeling studies during normal development and also in various pathologies. *In vivo* studies in human dental pulp have identified to date some proteoglycans such as decorin, biglycan and versican (Goldberg & Smith, 2004; Shibata, Kaneko, Yanagishita, & Yamashita, 1999; Yoneda, Shibata, Yamashita, & Yanagishita, 2002). Because dental pulp is an unmineralized tissue and is surrounded by dentin, which is a rigid mineralized one, it is relevant to study the components of the extracellular matrix, including proteoglycans, which is critical and well-known for bone mineralization. Besides, there is an increasing interest on dental pulp tissue engineering for regenerative purpose.

Within the last two decades, there have been few studies specifically investigating the lumican proteoglycan in human dental pulp. The distribution of this small leucine-rich proteoglycan (SLRP) was done in 1997 by Hall, Embery and Lloyd (Hall, Embery, & Lloyd, 1997) to understand the role of these macromolecules in the transition from predentine to dentine. This proteoglycan can modulate cell behavior, tissue repair and tumor growth as well as it regulates tissue hydration and collagen fibrillogenesis (Iozzo & Murdoch, 1996). Furthermore, the literature has not emphasized the importance of studying the six isoforms of glypican from the dental pulp. It is known that this proteoglycan is predominantly expressed during the development, suggesting its involvement in morphogenesis (Filmus, Capurro, & Rast, 2008). Associated to this scenario, there is no published data on proteoglycans lumican, versican and glypican in healthy human deciduous dental pulps. Thus, one question that needs to be asked is whether these specific proteoglycans are expressed in primary teeth and compare to the permanent ones.

Therefore, this *in vivo* study seeks to obtain data which will help address these research gaps. The primary aim of this investigation was to explore the presence of lumican, versican and glypican in human dental pulp, using real-time polymerase chain reaction (qPCR) from deciduous and permanent teeth. Secondarily, the lumican and versican proteoglycans were identified and compared by immunofluorescence analysis in these two conditions.

2. Materials and methods

2.1. Human dental pulps samples

Healthy human dental pulp samples were collected from teeth extracted for either orthodontic or occlusal reasons, as third molars, in general, do not reach functional occlusion with a significant pathologic potential. The procedures were performed based on the guidelines of the National Institutes of Health (NIH) regarding the use of human tissues and with permission from the Institutional Ethical Board from *Universidade Federal de São Paulo*, Brazil (protocol number 12636413.1.0000.5505). Terms of consent were obtained from adult patients and from parents in case of deciduous teeth after a brief explanation of the study.

Thirteen permanent normal dental pulps from premolar and third molar teeth, with closed apex, without cavities (clinically and radiographically examined) and restorations, were obtained from young adult patients of both genders, with no systemic diseases. Eight deciduous normal dental pulps from deciduous molars and deciduous canines, without cavities (clinically and radiographically examined) and restorations, were obtained from children patients from mixed dentition period, of both genders, with no systemic diseases.

In this study, human dental pulps from premolar and third molar teeth, as well as from deciduous molars and canines, were preferable to supernumerary teeth as the etiology of the Hyperdontia is still not totally understood (Inchingolo et al., 2010). Thus, dental anomalies were avoided in the samples.

The operative procedures to extract the teeth, in dental private practice, included disinfection with 2% chlorhexidine digluconate and local anesthesia (2% noradrenalin; Scandicaine; Septodont, Saint-Maur-Des-Fosses, France). After teeth extraction, cavities were performed with carbide burs under air-distilled water cooling to approximate the pulp chamber. The pulp was gently removed using a dental scoop, without demineralization of the teeth. For deciduous teeth, the pulp was directly removed after extraction because the roots were already reabsorbed.

2.2. Real-time PCR

Three permanent and three deciduous dental pulps were extracted, as described previously, and stored in a pre-cooled RNase-free microcentrifuge tube to be transported immediately to the laboratory on dry ice. The dental pulp was firstly macerated with 500 µL of TRIzol® reagent (Invitrogen, Life Technologies Corporation, CA, USA) and the RNA extraction was accomplished following the manufacturer's protocol. Extracted RNA was solubilized in 40 µL of RNase-free water and quantified by spectrophotometry at 260 nm. The total RNA was then subjected to RT-PCR for cDNA synthesis. Versican, lumican and glypican gene expression were quantitatively evaluated by real-time PCR technique, using as control the expression of the endogenous gene GAPDH. Real-time PCR was performed with the SYBR® Green PCR Master Mix, including AmpliTaq-GOLD polymerase on ABI PRISM 7500 Real Time PCR System (Applied Biosystems, USA). All reactions were performed in triplicate. The primers used for the amplification reaction were designed from research database sequences. The nucleotide sequences are shown in Table 1.

The reaction was performed as follows: 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C, followed by 30 s at 72 °C. We plotted a standard curve for each primer by applying known quantities of the PCR products of each sample. The expression levels of versican, lumican and glypican were normalized to the GAPDH gene expression level.

2.3. Immunofluorescence

Ten permanent and five deciduous dental pulps were extracted, immediately stored in 10% paraformaldehyde (PFA) and transported to the laboratory. The specimens were kept for 24 h in this solution and dehydrated in 30% sucrose for 24 h. The specimens were then embedded in Tissue Freezing Medium (Tissue-Tek® O.C.T. Compound, Sakura®Finetek) and frozen at −80 °C. The frozen specimens were cut (Cryostat/Leica CM 1850) in serial sections at a thickness of 25 µm, captured on glass slide properly identified, and stored at −20 °C for staining. Prior to the immunohistochemical staining, some sections were stained with hematoxylin-eosin (HE).

The following sequence was carried out to perform the immunofluorescence. The glass slide chosen was kept at room temperature for 15 min. The sections were fixed in 4% PFA prepared in phosphate-buffered saline (PBS) for 20 min, washed three times in PBS for 5 min each, and incubated with blocking buffer containg 10% fetal bovine serum (FBS) diluted in PBS and 0.1% Triton X-100 detergent for one hour. Afterwards, the sections were incubated with the primary antibodies overnight at 4 °C. All primary antibodies were diluted in 10% PBS with 0.1% Triton X-100 at concentrations described in Table 2.

The sections were washed three times in PBS for 5 min and followed by incubation with secondary antibody (1:100) for one hour at 4 °C, in dark room. Actin filaments were stained with Alexa 488-conjugated phalloidin (1:500, Invitrogen) for 30 min. The sections were washed again in PBS for 5 min and DAPI (4′,6-diamidino-2-phenylindole),

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