In vitro immunoexpression of extracellular matrix proteins in dental pulpal and gingival human fibroblasts

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Abstract

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Aim To compare the expression of extracellular matrix (ECM) components in human pulpal and gingival fibroblasts *in vitro*.

Methodology Cultured dental pulp fibroblasts and gingival mucosa fibroblasts were used. Tenascin (TN), fibronectin (FN), type I (col I) and III collagen (col III) and osteonectin (ONEC) were detected by immunofluorescence. Main morphological characteristics were also analysed by light microscopy (LM) and transmission electron microscopy.

Results The results revealed different expression patterns of the proteins. TN and ONEC were only immunoexpressed by pulpal fibroblast cells, suggesting a role of these glycoproteins in formation of mineralized tissues. FN and col I were present in the cytoplasms of both cell types. No expression of col III was detected. Different morphological characteristics were visualized under LM, in which pulpal fibroblasts were spindle-shaped with a wide cytoplasm, while gingival fibroblast cells exhibited stellate/pyramidal configuration, with rounded nuclei. However, ultrastructurally, both cell lineages showed very well developed rough endoplasmatic reticulum and Golgi complex.

Conclusions Due to the immunodetection of TN and ONEC on pulpal fibroblasts, the present findings demonstrated that a pulpal fibroblast cell is similar to an osteoblastic cell rather than an undifferentiated mesenchymal cell, such as a gingival fibroblast cell. Functional differences between the two cell lines may then be suggested.

Keywords: dental pulp, extracellular matrix proteins, gingival fibroblasts, human dental pulp fibroblast.

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Introduction

The dental pulp is a specialized loose connective tissue composed of cells and extracellular matrix (ECM). Whereas the periphery of the pulp is formed mainly by odontoblasts, its central region contains a heterogeneous population of cells, which includes stromal, endothelial, neural, immune cells and others. However, the main cell type of dental pulp is the fibroblast, responsible for the synthesis and secretion of the ECM (Goldberg & Lasfargues 1995).

The ECM comprises a variety of proteins and polysaccharides that are secreted locally, forming an organized network. The main types of matrix macromolecules that constitute the human dental pulp are collagenous proteins [type I (col I), III and IV collagens], noncollagenous proteins [fibronectin (FN), tenascin (TN), osteonectin (ONEC), osteopontin and osteocalcin], proteoglycans (chondroitin sulphate and heparan sulphate) and phospholipids (Goldberg & Lasfargues 1995). The ECM serves not only as a scaffold to stabilize its physical structure (Van Amerongen *et al.* 1983, Karjalainen *et al.* 1986,

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Hillmann & Geurtsen 1997), but it has also an active role in influencing the development, migration, proliferation, shape and function of all the pulpal cells (Lesot *et al.* 1981, Lukinmaa *et al.* 1991, Chiquet-Ehrismann *et al.* 1994). Therefore, alterations in these relations may interfere with the integrity of the tissues.

Tenascin is a large oligomeric glycoprotein of the ECM which is secreted by fibroblasts and glial cells in tissue culture. It is a disulphide-linked hexamer with subunit molecular weight, which ranges from 190 to 320 kDa. During embryogenesis, this glycoprotein is transiently expressed in the dense mesenchyme surrounding developing organs such as the mammary gland, teeth, kidney, embryonic cartilage and nervous system. In adults, this protein becomes confined into tissues submitted to mechanical loads such as, pericondrium, periostium, ligaments, tendons, myotendinous junctions and smooth muscle. It is also found in malignant epithelial and mesenchymal tumours and healing wounds (Chiquet-Ehrismann 1990, 1995). The TN expression is also regulated by growth factors as TGF-β (Erickson & Bourdon 1989, Chiquet-Ehrismann 1990) and by mechanical stress (Chiquet-Ehrismann *et al.* 1994, Chiquet *et al.* 1996). In dental tissues, TN has been shown to be important in the differentiation of odontoblasts (Thesleff *et al.* 1987). Therefore, it may be associated with secondary dentine elaboration, when pulp cells differentiate into odontoblasts in response to physiological stimuli (Thesleff *et al.* 1987, 1991, Lukinmaa *et al.* 1991, Martinez *et al.* 2000).

Fibronectins are a class of high molecular-weight adhesive proteins (220 kDa) composed of two very large subunits joined by a pair of disulphide bonds. There are multiple forms (isoforms) of FN, including plasma FN, which is soluble and circulates in the blood and other body fluids. All the other forms assemble on the surface of cells and are deposited in the ECM as the highly insoluble FN (Martinez-Hernandez & Amenta 1983). Many cell types produce FN, including epithelial, endothelial and mesenchymal cells (Ruoslahti 1981). Fibronectin has been implicated in a variety of cell functions including adhesion, migration, growth and differentiation (Yamada & Olden 1978, Ruoslahti 1981). FN is also found in association with dental basement membrane during tooth formation and may play a crucial role in the migration, polarization and differentiation of odontoblasts (Lesot *et al.* 1981).

The collagens are a family of fibrous proteins found in all multicellular animals, constituting 25% of their total protein mass. In human dental pulp, col I and col III constitute the bulk of the tissue collagen. Type III collagen constitutes a large proportion (43%) of the total collagen (Van Amerongen *et al.* 1983) and may have an important function in the elasticity of the tissues (Shuttleworth *et al.* 1980). In contrast, col I is thought to maintain tissue architecture (Narayanan & Page 1983). The collagens are also the main organic component found in dentine (80–90%) (Linde 1985). However, no col III is found in dentine (Scott & Veis 1976).

Osteonectin is the major noncollagen protein of bone, having a molecular weight of 32–46 kDa (Fisher & Termine 1985), and is responsible for the mineralization properties of this tissue (Termine *et al.* 1981). This protein is an avid binder for hydroxyapatite and collagen and promotes the deposition of calcium phosphate mineral onto col I. Thus, it has been proposed that ONEC may play a role in bone mineralization *in vivo* (Ingram *et al.* 1993, Robey 1996).

Due to the great importance of the ECM on tissue development and cellular behaviour, the aim of this study was to analyse by immunofluorescence the expression of ECM proteins (TN, FN, col I, col III and ONEC) in cultured fibroblasts from the human dental pulp. For comparison, cultured fibroblasts from gingival mucosa were studied. In addition, we also examined the main morphological features of these cells under light microscopy (LM) and transmission electron microscopy (TEM).

Materials and methods

Cell cultures

The pulpal fibroblasts were obtained from a human third molar germ extracted for orthodontic reasons and gingival mucosa fibroblasts, from explants of healthy attached human gingiva. These tissues were used with the patient's informed consent and appreciation of the Research Ethics Committee of the University of São Paulo.

The cells were then cultured in Dulbecco's modified Eagle's medium (DMEM®) (Sigma, St Louis, MO, USA) supplemented with 1% antimicotic solution (Sigma), containing 10% foetal bovine serum® (Cultilab, Campinas, SP, Brazil). The cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2/95% air, supervised every 24 h. When the cells reached subconfluence, they were harvested with trypsin and

subcultured. The fibroblast cell cultures used in the following procedure were from passages three to six for all experiments.

Immunofluorescence

Cells grown on coverslips were fixed in 1% paraformaldehyde in phosphate-buffered saline solution (PBS) for 10 min, rinsed in PBS and permeabilized with 0.5% Triton X-100 solution for 10 min at room temperature. The cells were incubated with anti-TN® (DAKO, Carpinteria, CA, USA), anti-FN® (DAKO), anti-col I® (NOVO CASTRA, Newcastle, UK), anti-col III® (DAKO) and anti-ONEC (LF-37) (Fisher *et al.* 1995), kindly supplied by Prof. Dr Larry W. Fisher (NIDR, NIH, Bethesda, MD, USA). In addition, the cells were incubated with anti-vimentin (VIM®) (DAKO), to certify the mesenchymal origin of the studied cells. The primary antibodies were used at 1 : 50 dilution in PBS and 1% bovine serum albumin (BSA), for 1 h, at room temperature. The coverslips were rinsed in PBS and anti-rabbit immunoglobulins coupled to fluorescein® (DAKO) were used as secondary antibody for the detection of FN, col I and ONEC. Anti-mouse immunoglobulins coupled to fluorescein® (DAKO) were used to detect TN, col III and VIM. The coverslips were rinsed in PBS and in distilled water, and mounted in Vectashield® (Vector Laboratories Inc., Burlingame, CA, USA).

The expression of the studied proteins was carried out using a Zeiss Axiophot 2 fluorescence microscope with 63× Plan Apochromatic 1.4 NA and 100× Plan Apochromatic 1.4 NA objectives in standard conditions (Carl Zeiss, Oberköchen, Germany).

Light microscopy and transmission electron microscopy

For LM, the cells were fixed with 1% paraformaldehyde at 4 °C, treated with triton X-100® (Sigma), and stained with haematoxylin for 15 min, followed by eosin for 20 min, at room temperature. The coverslips were mounted in Permount® (Fischer Chemicals, Fair Lawn, NJ, USA), and the morphological features of both cell types were observed with a light microscope.

For TEM, the cells were fixed in 2% glutaraldehyde buffered with 0.1 mol L^{-1} sodium phosphate (pH 7.4) for 2 h at room temperature. Then, the cells were post-fixed with 2% osmium tetroxide for 30 min, dehydrated in a graded series of ethanol and embedded in Epon. The cell apparatus was examined by ultra-thin (80 nm) sections under a JEM 1010 transmission electron microscope (Jeol USA Inc., Peabody, MA, USA) operating at 80 kV.

Results

Immunofluorescence

Both cell lineages showed a very strong labelling for VIM. It exhibited a reticular network-like appearance in the cytoplasm (Fig. 1a,d).

Fibronectin and col I were present in the cytoplasm of both cell types (Fig. 1). Type I collagen labelling was present throughout the cytoplasm of both cells, forming vesicles that appeared concentrated around the nuclei (Fig. 1b,e). FN exhibited an intense immunostaining in the cytoplasm with a reticular network (Fig. 1c,f).

Tenascin and ONEC were only expressed on pulpal fibroblasts (Fig. 1). TN showed a diffuse reticular network staining (Fig. 1g). ONEC was expressed throughout all the cytoplasm of the pulpal fibroblasts (Fig. 1h).

No expression of col III was found.

Light microscopy

Different morphological characteristics were visualized under LM. The pulpal fibroblasts were spindle-shaped with a wide cytoplasm, whereas the gingival fibroblasts exhibited a stellate appearance with rounded nuclei, occasionally with a pyramidal shape (Fig. 1i,j).

Transmission electron microscopy

The ultrastructural examination revealed numerous cisterns of rough endoplasmic reticulum and a well-developed Golgi complex in both the cell lineages, with the presence of some mitochondria and lysosomal vacuoles. The microfilaments were also evident in both cell types, exhibiting a network-like appearance throughout the cytoplasm (Fig. 2).

Discussion

The present study showed that cultured pulpal and gingival fibroblasts had a different expression pattern of the ECM proteins studied.

Both cell lineages exhibited immunostaining for VIM demonstrating the mesenchymal origin of these cells. A variety of cells of mesenchymal and nonmesenchymal origin, as well as all cell types grown in tissue culture, contain a class of intermediate filaments, known as vimentin filaments. The functions of VIM filaments are

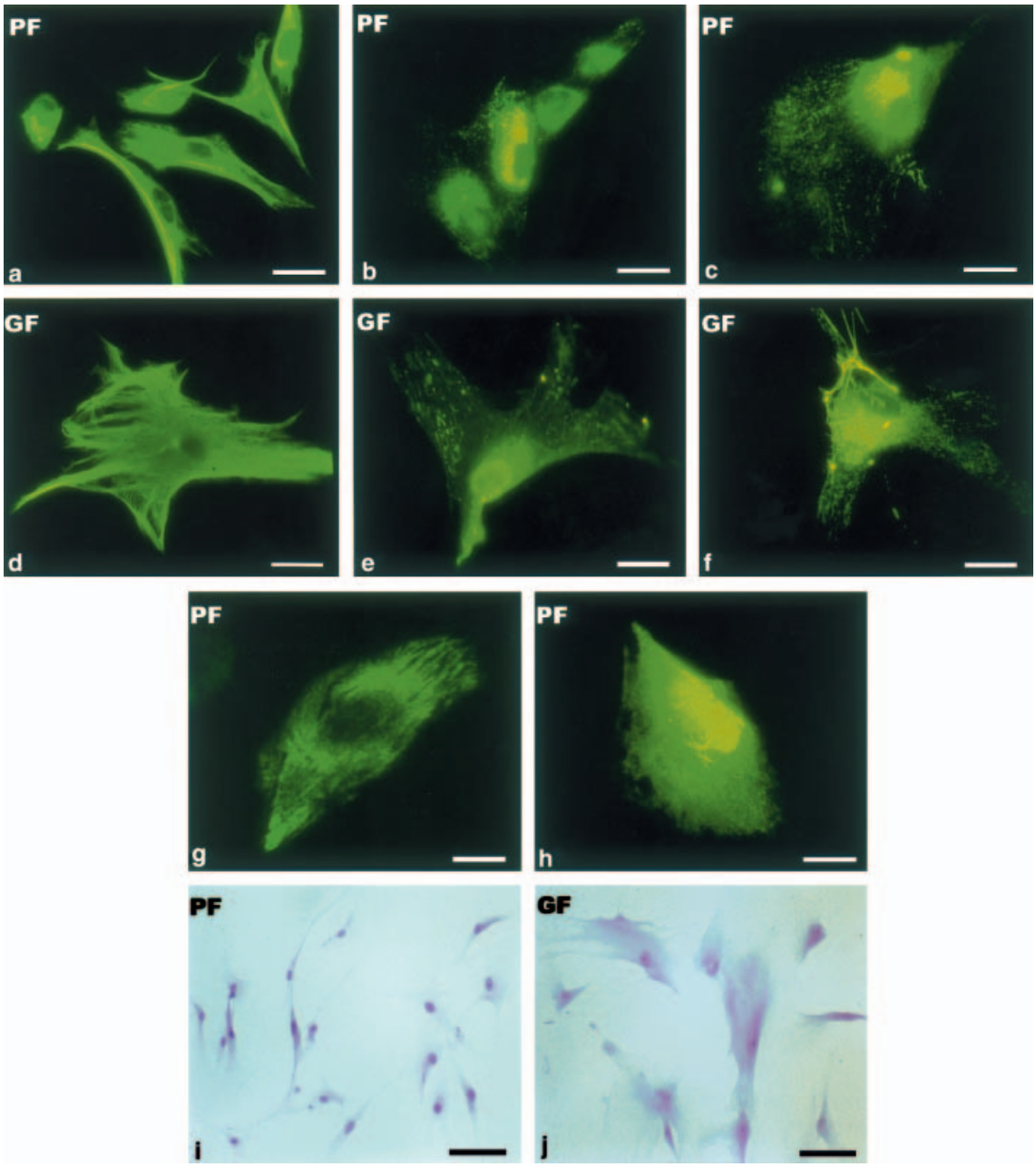

Figure 1 Immunofluorescent staining for VIM (a–d), type I collagen (b–e), FN (c–f), TN (g) and ONEC (h) on pulpal (PF) and gingival fibroblasts (GF). Note the similar reticular network staining for VIM (a–d) and FN (c–f). Type I collagen is present diffusely in the cytoplasm, mainly in the perinuclear region, forming vesicles (b–e). TN shows a diffuse reticular network staining (g). ONEC is expressed throughout the pulpal fibroblast cytoplasm (h). Haematoxylin–eosin staining (H & E) of pulpal and gingival mucosa fibroblasts (i–j). Pulpal fibroblasts show wide cytoplasm with a spindle-shaped appearance (i), while gingival fibroblasts exhibit stellate configuration, occasionally pyramidal, with rounded nuclei (j). Bars: a–h = 150 μm; i = 100 μm; j = 50 μm.

poorly understood, but are presumed to be structural due to the insolubility properties of VIM filaments (Lazarides 1980, 1982).

In the present study, TN and FN were present in pulpal fibroblasts. TN and FN are prominently expressed in embryonic basement membranes, tumour

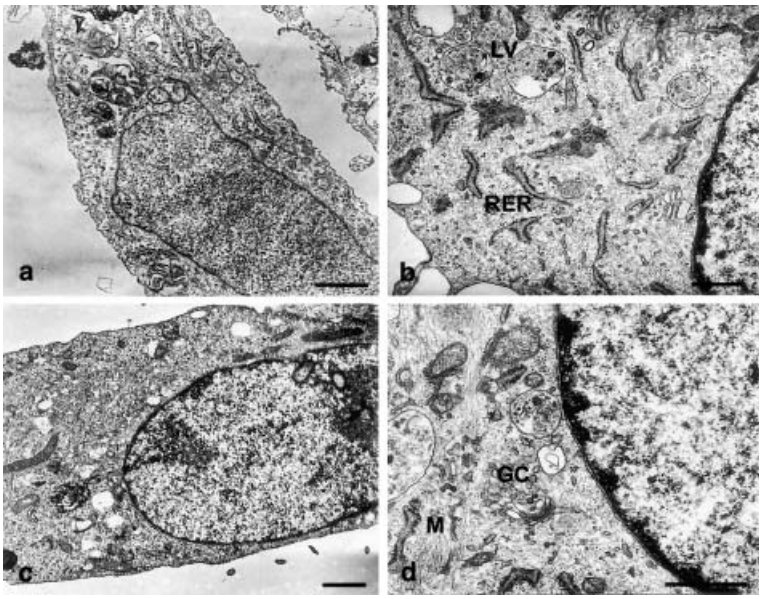

Figure 2 Transmission electron micrographs of pulpal (a, b) and gingival mucosa fibroblasts (c, d). Both cell lineages exhibit well-developed rough endoplasmatic reticulum (RER) and Golgi complex (GC). In addition, the cells show lysosomal vacuoles (LV). Note the presence of numerous microfilaments (M) spread like a network throughout the cytoplasm. Bars: a, c and d = 1 μm; b = 0.5 μm.

stroma and granulation tissue, involved in differentiation and/or growth (Thesleff *et al.* 1987, Chiquet-Ehrismann 1990). In the dental basement membrane, TN and FN have been suggested to trigger odontoblast differentiation (Lesot *et al.* 1981, Ruch 1998), a process associated with changes in the expression of ECM components.

During odontogenesis, dental mesenchymal cells secrete TN and FN, which are believed to be involved in the alignment and differentiation of the dental papilla cells into odontoblasts (Lesot *et al.* 1981, Ruch 1998). In contrast, when this differentiation is complete, dental mesenchymal cells cease to express FN and TN (Thesleff *et al.* 1987). The maintenance of TN and FN expression in adult human dental pulp appears to be associated with regulation of cell differentiation involved in the formation of hard tissues matrices (Thesleff *et al.* 1987, Lukinmaa *et al.* 1991, Martinez *et al.* 2000).

When pulpal injury occurs, the odontoblasts are stimulated, to form tertiary dentine. If the odontoblasts are destroyed, the repair is associated with new odontoblasts differentiated from mesenchymal cells, even without the basement membrane. The ECM is likely to influence this process due to the presence of TN and FN. In this context, it is conceivable to consider that the maintenance of TN and FN in pulpal fibroblasts could be associated with a function of these cells as precursor cells for hard tissues matrices. However, studies have demonstrated that the regulation of TN synthesis is mediated by mechanical stimuli (Chiquet *et al.* 1996). It can be suggested that TN synthesis in pulpal fibroblasts may be related to mechanical stress, as environmental factors may alter the cellular and extracellular components (Chiquet-Ehrismann 1990, 1995). In contrast, gingival fibroblasts did not express TN. Although TN is important for cell migration as in wound healing (Martin 1997), it exhibits a limited distribution in adult tissues, and seems to be involved in the formation of hard tissue matrices (Thesleff *et al.* 1987, Lukinmaa *et al.* 1991, Martinez *et al.* 2000) and mechanical loading (Chiquet *et al.* 1996). However, FN influences the migration of fibroblasts and plays a crucial role in maintaining structural integrity of connective tissues (Palaiologou *et al.* 2001). Baum & Wright (1980) demonstrated the presence of FN in cultures of human gingival fibroblasts, and considered a role for FN in the organization of gingival tissues. It also has great importance during endothelial regeneration and may constitute angiogenic elements in neoformed tissue (Wegrowski *et al.* 1989). Thus, FN immunostaining in gingival fibroblasts, indicates a possible role of this protein in gingiva by mediating adhesion, migration and cell proliferation.

The presence of ONEC only in pulpal fibroblasts suggests a probable role of this glycoprotein, as suggested for TN, in mediation and formation of mineralized tissues. ONEC is a bone-specific protein, playing a key role in bone formation by initiating

mineral deposition and by linking mineral to matrix (Termine *et al.* 1981). Although ONEC was thought to be unique to bone and mineralizing connective tissue matrices, it has been also found in periodontal ligament fibroblasts (Wasi *et al.* 1984) which can differentiate into osteoblasts or cementoblasts. Similarly, pulpal fibroblasts can differentiate into odontoblasts, in response to physiological stimuli, to form tertiary dentine.

Tenascin and ONEC, proteins responsible for the hard tissue mineralization, were only expressed in pulpal fibroblasts, suggesting that pulpal fibroblasts are more similar to osteoblasts than to undifferentiated mesenchymal cells.

Whereas col I was expressed by both cell lineages, there was no immunoexpression of col III in none of the studied cells. It has been suggested that treatment of cells with proteolytic enzymes, for instance trypsin digestion, leads to a greater degradation of col III relative to col I (Conrad *et al.* 1980, Van Amerongen *et al.* 1983). The possibility exists that the change in the cellular phenotype may be caused by alterations of the cell surface as a consequence of protease activity (Conrad *et al.* 1980). The intensity of fluorescence found intracellularly does not give any information about the amount of collagen produced. Most of the collagen produced *in vitro* is secreted as a soluble precursor (procollagen) into the medium. Hence, the presence of col I in both cell types is associated with the tissue architecture.

The different morphological patterns between pulpal and gingival fibroblasts, as visualized under LM, are related to functional differences of the cells studied. Comparing with other cell types (Lilla *et al.* 2002), it is reported that cell shape changes are crucial to differentiation, and that these changes in shape are modulated by ECM components. Thus, it is suggested that even *in vitro* conditions, each fibroblast has a specific morphological pattern to secrete different ECM proteins, being responsible for the change and maintenance of cell shape. However, the two cell lineages were ultrastructurally similar, containing well-developed rough endoplasmatic reticulum and Golgi complexes. This finding shows that these cells have the capacity to synthesise and secrete ECM proteins.

Conclusions

Pulpal and gingival human fibroblasts have a different expression pattern of ECM *in vitro*. Due to the immunodetection of TN and ONEC on pulpal fibroblasts, the present findings demonstrated that a pulpal fibroblast cell is similar to an osteoblastic cell rather than an undifferentiated mesenchymal cell, such as a gingival fibroblast cell. This may reflect a functional difference between these cell types.

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