Journal of Oral Research

ORIGINAL ARTICLE

Transforming Growth Factor -ß1 (TGF-ß1) immunoreactivity in heterotopic grafts of adult dental apical papilla.

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Receipt: 12/11/2014 **Revised:** 12/23/2014
Acceptance: 01/27/2015 **Online:** 01/27/2015

Abstract: To analyze the expression of transforming growth factor-ß1 in heterotopic grafts of adult dental apical papilla. Methodology: The apical papilla of adult Wistar rats was grafted in the ear of the same donor rats.1, 3, 7 and 14 days after grafting, rats were perfused and the tissue containing the graft was processed for histological conventional technique and for immunohistochemical detection of transforming growth factor-ß1. Results: Heterotopically grafted apical papilla developed osteoid dentine. In an early post-grafting stage, odontoblast-like cells organized themselves in palisade and synthesized dentine. However, newly formed dentine possessed the structural appearance of reactive osteoid dentine, which was systematically destroyed by the activity of osteoclaste-like cells. Transforming Growth Factor-ß1 was observed in mesenchymal cells, extracellular matrix of the graft and surrounding host tissue, while odontoblast-like cells were systematically devoid of immunoreactivity. Conclusion: The different expression of transforming growth factor-ß1 between normal tissue and grafted tissue development suggests that in heterotopic graft conditions the inflammatory mediation of the transforming growth factor-ß1 prevails against its morphogenetic role

Keywords: *Odontoblast, Apical papilla, TGF-ß1, Graft.*
DOI: *10.17126/joralres.2015.009*

Cite as: *Mezadri T, Tames D, Ortolan X & Armengol J. Transforming Growth Factor -ß1 (TGF-ß1) immunoreactivity in heterotopic grafts of adult dental apical papilla. J Oral Res 2015; 4(1):38-43.*

INTRODUCTION.

Dental pulp contains adult stem cells or stem cells which differentiate into odontoblast-like cells and produce, both in transplants and in cultivation, a dentin-like tissue[1]. The progenitors of these cells come from the neural crest and colonize the dental papilla during odontogenesis[2]. The potential of these ectomesenchymal stem cells opens a range of therapeutic treatments in advanced restorative dentistry, for which the autologous or allogeneic transplant of these cells would allow for the regeneration of adult dental and periodontal tissues[3-6].

Odontogenesis is regulated by complex cascade molecules. Among the molecules that regulate the final stages of odontogenesis, the transforming growth factors superfamily (TGF) stands out, specially the transforming growth factor-beta[1] (TGF-ß1). It is involved in several stages of embryogenesis such as dental amelogenesis, controlling the expression of matrix metalloproteinases[7-8], in cementogenesis and the regeneration of adult periodontal tissue[9], in the differentiation of the odontoblasts and secretion of the predentina[10]. Differentiated odontoblasts secrete TGF-ß1 and regulate its activity in an autocrine manner[11]. Therefore, errors in TGF-ß signaling alter odontoblast differentiation and dentin formation directly or through the transcription factor SMAD4[12].

The incisive of rats contains stem cells in the apical papilla of its root which allow a continuous tooth growth. This growth is regulated in two ways. On one hand, the

molecular interactions between the cervical epithelium and the dental papilla allow forming enamel coated dentin in the labial region and cement coated dentin and periodontal ligament in the lingual area[13]. On the other hand, TGF-ß regulates the growth of the epithelial stem cells with the purpose of maintaining the continued growth of the incisive, becoming a source of ectomesenchymal stem cells for study models that can help to understand dental tissue repair[14].

Stem Cells of dental origin are used in the search of new therapeutic approaches with the aim of promoting tissue regeneration in the case of loss of pulpal and dentinal tissues[3-6,15].

The formation of tertiary dentin, mainly characterized by cellular inclusions and called osteoid dentin because of it, is a constant factor in the development of dental embryo transplants at an advanced stage of odontogenesis, dental papilla and stem cells[12,15-17]. And, despite knowing the functions that TGF-ß1 plays in odontoblastic differentiation and dentinogenesis, very little is known about its participation in the formation of tertiary dentin, both as a response and in restoration. This qualitative descriptive study analyzes the evolution of the immunohistochemical expression of the TGF-ß1 protein in the apical papilla of the incisive of adult rats heterotopically transplanted.

METHODOLOGY.

The transplant procedure has previously been described[16]. The apical papilla was obtained from adult incisors of 60-days-old Wistar male rats. They were handled under the criteria of the directive 2010/63/EU- in accordance with the principles of animal welfare - (there was neither loss nor abnormal behavior of the rats during the experiment). Experimental animals were anesthetized with a mixture of ketamine hydrochloride (Agener®, Agener Uniao, SP, Brazil) and xylazine hydrochloride (Calmiun®, Agener Uniao, SP, Brazil) (80mg/kg-10mg/kg, i.m.). The dental papilla was removed with forceps and washed with cold surgical saline in a Petry plate and immediately transplanted into a subcutaneous pocket made in the skin of the ear of the same animal. We used a total of 12 rats that were euthanized in groups of three: 1, 3, 7 and 14 days post-transplantation (DPT). They were anaesthetized with the same mix and transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer. Tissue samples containing the transplant were removed and then fixed in the same solution at 4ºC for 24h, demineralized with EDTA during one week, dehydrated in alcohol baths of increasing concentration (70, 80 and 100%), clarified in xylol baths, impregnated and embedded in paraffin. Sections (7 µm thick) were processed in two series. One was stained with hematoxylin-eosin and the other was used for conducting immunohistochemistry of TGF-ß1 according to the manufacturer's instructions (TGF-ß1 SC 146, Santa Cruz Biotechnology® INC. CA, USA) and revealed with DAB as a chromogen. The sections were slightly stained again with a toluidine blue 1% aqueous solution. As control of the immunohistochemical reaction, cuts were processed with omission of the primary antibody without marking for each series. All the samples were observed in a photomicroscope (BX-50F4, Olympus®, Japan).

RESULTS.

Before the transplant, the apical papilla is a mesenchyma formed by undifferentiated cells (Fig 1A) among which the expression of TGF-ß1 is detected (Fig 1B). From 1 DPT, the apical papilla showed consistent signs of tissue differentiation in the alignment of odontoblast-like cells without signs of matrix secretion (Fig 1C), and the TGF-ß1 immunohistochemical reaction in the extracellular matrix was similar prior to transplantation (Fig 1D). During this time, the transplanted tissue and the host showed inflammatory signs of infiltrate of leukocytes and edematous extracellular space (Fig 1C).

After 3 DPT, the transplant developed a structure that resembles the embryonic tissue of the apical papilla (Fig 2A, PA); however, the isolation of the host tissue is not complete and it is common to find inflammatory

infiltrates in adjacent areas between both tissues (Fig 2A). (Fig 2A-B) Odontoblastic-like cells, which are organized in palisade and secrete dentin, were observed in the periphery of the transplant (Fig 2A-C). Dentin is deposited in a layer upon the apical surface of the cells imitating its normal polarization in the tooth (Fig 2C). The dentin of the transplant has structural features which are characteristic of the tertiary or osteoid dentin such as: (i) fewer numbers of dentinal tubules with disorderly distribution (Fig 2C), and (ii) loss of the stratified structure of the peripheral region of the normal pulp, causing a hard tissue that includes odontoblastic cells islet that secrete it (Fig 2C). The host tissue that surrounds the transplantation showed inflammatory signs as engorged vessels and lymphoid cells (Fig 2D). Immunoreactivity to TGF-ß1, absent in the odontoblasts, was observed in the matrix of the transplant around their mesenchymal cells (Fig 2E-F) and in the receptor tissue next to the transplant (Fig 2G).

The disorganization of the transplant was most apparent 7DPT and although areas with odontoblastic cells in palisade and covered by the layer of dentin are preserved (Fig 3C), most of the osteoid dentin had increased its size including odontoblastic cells islet (Fig 3B-C), while the organization in palisade decreased (figure 3C) until it disappeared (Fig 3B). Like in the early transplants (1 and 3 DPT), the inflamatory reaction persisted (Fig 3A) at the same time that the immunoreactivity to TGF-ß1, still present in some mesenchymal cells, was more marked in the matrix of the transplant (Fig 3D-E).

Fourteen DPT, almost all of the transplant was made up of osteoid dentin with islets cells and without evident cellular organization in palisade (Fig 4A). The interior of these lacunes presented evidence of osteoclastic activity, such as the presence of multinucleated cells with vacuoles which are reminiscent of the assets of the osteoclast bone (Fig 4B, arrows). Immunoreactivity to TGF-ß1 was spread through the entire array (Fig 4D) and few cells have a very discreet positive immunoreactivity in the cytoplasm (Fig 4C).

Figure 1. Photomicrographys of adult apical papilla before (A-B) and 1 DPT (C-D). Note the undifferentiated aspect before transplantation (A). TGF-ß1 is evident in mesenchymal cells (B, arrows) and in the extracellular matrix (B, C). After the transplant, cells begin to organize in a layer (C, c). Both the transplant (PA) and the host tissue have lymphoid cells (C, arrows) and inflammatory signs. TGF-ß1 is discreetly evident in the cells of the mesenchyma of transplant (D, arrow). PA, apical papilla e, edema. V, blood vessels. A and C, hematoxylin-eosin. Bars = 50 µm (A) and 20 µm (B-D).

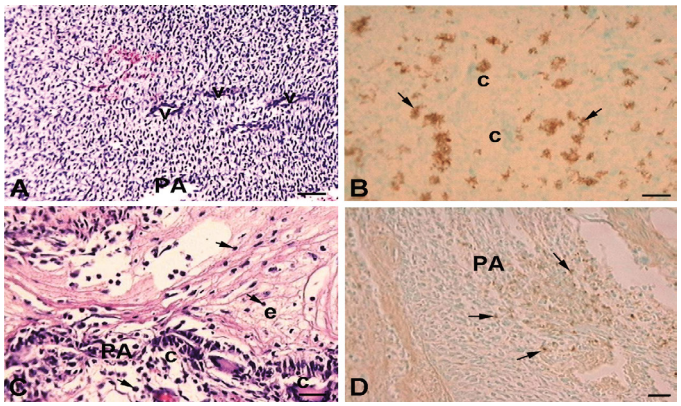

Figure 2. Photomicrographys of transplants of adult apical papilla 3 DPT. Transplantation has odontoblastics cells that are organized in palisade (A-C, arrows) and secrete dentin (A-B, D). The dentin of osteoid matrix (C, m,) has disorganized dentinal tubules (C, arrow heads) and includes islet (C, ic). The host tissue has dilated vessels (D, v) and lymphoid cells (D, arrow). TGF-ß1 is evident in the cells of the mesenchyma of transplant (E-F, arrows) and the receiver (G, arrows); but it is absent in odontoblastic cells. AP, transplanted apical papilla. i, inflammatory host interface-transplant. A-D, hematoxylin-eosin. Bars = 50 µm (A), 30 µm (B and D), 20 µm (C, E and G) and 10 µm (F).

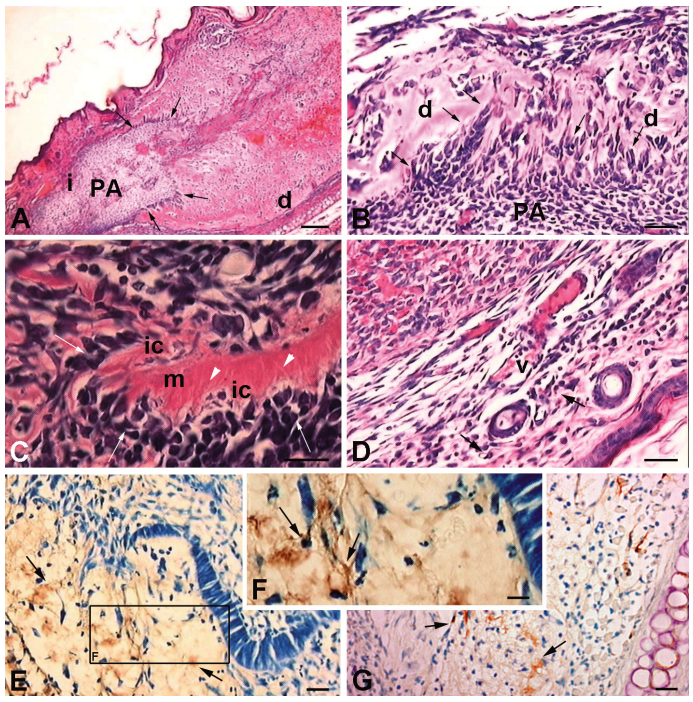

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Figure 3. Photomicrographs of transplants of apical papilla 7 DPT. The growth of the osteoid dentin (d) breaks the organization of odontoblastic cells in palisade (A), forming islets cells (B-C, arrows). Note the absence of palisade in B (arrow heads) and the inflammatory reaction in host (i). TGF-ß1 is evident in cells and in the matrix of the transplant (D-E). PA, apical papilla. A-C, hematoxylin-eosin. Bars = 50 µm (A-B and D) and 20 µm (C and E).

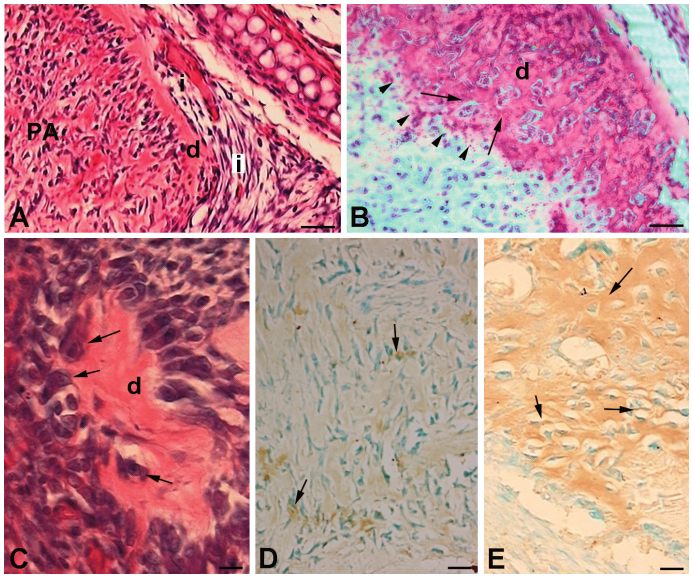
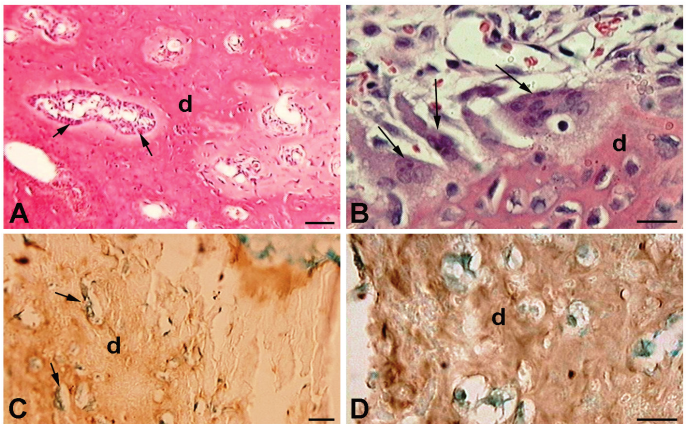


Figure 4. Photomicrographs of transplants of apical papilla 14 DPT. Osteodentin occupies almost the entire transplant (d) and in its interior there are gaps of cells (A, arrows) and osteoclastic activity (B, arrow). Immunoreactivity to TGF-ß1 is evident in the matrix (C-D) and is occasionally very weak in some cells (C, arrows). A and B, hematoxylin-eosin. Bars = 50 µm (A) and 20 µm (B-D).



DISCUSSION.

The capacity of the apical papilla to generate dental structures has been described in various experimental trasplant conditions[16,18]. Today, this capability is explained by the presence of pluripotent stem cells in the adult dental pulp[2,4]. These stem cells are capable of differentiating into secretory cells of dental tissue, including dentin secreting odontoblasts[1]. One of the most striking and constant features is the development of a layer of polarized odontoblastic cells, on whose apical surface the secreted dentin is placed[1,16,18]. Since the first transplants of dental papilla to the present time, there are two key and repeated questions: (i) what are the factors involved not only in the development of the transplant but also in its interaction with the receptor? and (ii) what is the actual time duration of the transplant within the host? and, therefore, what is the potentiality of the transplant in relation to dental tissue regeneration?

In an attempt to avoid the possible deleterious effects of the reaction of the host in the transplant, the strategy of performing heterotopic transplants of matrix dentin proteins[19], embryonic tissue[16,20] and stem cells previously isolated and amplified in culture in isogenic or immunosuppressed animals has been used from the beginning[1]. Dentinogenesis was always obtained in these conditions[1,16,18,20]; however, it is not yet clearly elucidated how stable this dentin is with time. Our data demonstrate[16] cells in the transplant developed reactive tertiary or osteoid dentin occupying the entire transplant during the first week after transplantation and it was finally eliminated. This type of dentin is characterized by the loss of its tubular structure and the acquisition of a morphology similar to the bone has been described in various clinical and experimental conditions[21,22], among them the trasplants[10,16,20], and its appearance has been justified as the result of a reaction to exogenous factors, among which is the inflammatory reaction of the host[16].

The family of bone growth regulatory proteins TGF-ß, in addition to its morphogenetic role, has been implicated in other pathological processes such as inflammation, fibrosis, cancer and early cell death[23,24]. The molecules involved in dental development have, among other functions: the growth of the dental embryo[7,8,26]; promoting odontoblast differentiation and matrix secretion[16,25,27] with TGF-ß1 expression and its receptor in the

odontoblasts[8]; and the control of the number of ameloblasts through apoptosis during the final phase of the amelogenesis[28]. In our experiments and unlike the normal development, TGF-ß1 was not expressed in the odontoblasts of the transplanted apical papilla but in the mesenchymal cells of the transplantation and in the surrounding tissue of the host. This abnormal TGF-ß1expression, coupled with ectopic induction of TGF-ß1 in the tissues of the host, probably as a result of the inflammatory response after transplant, may indicate that TGF-ß 1 would act, in this case, by enhancing the phenomena of cell death, as ameloblasts do when their protein is overexpressed[29].

In all the cases described above, including the present experiments, most of the dentin produced has morphological characteristics of osteoide dentin[1,16,19,20]. Thus, while stem cells cultivation for production of dentin *in vitro* for further applications opens a promising biological perspective[1], our results show that differentiated odontoblasts in the transplant did not survive beyond two weeks and suggest that TGF-ß1 participates more as a promoter of the inflammatory process than as a regulator of odontogenesis transplantation.

Inmunoreactividad del Factor Transformador del Crecimiento-ß1 (TGF-ß1) en la papila apical dental adulta heterotópicamente trasplantada.

Resumen: Objetivo: Analizar la expresión del factor transformador del crecimiento-ß1 en trasplantes heterotópicos de papila dental del incisivo de la rata adulta. Metodología: La papila apical del incisivo de 12 ratas Wistar adultas fue trasplantada en la oreja de las mismas ratas donantes, y perfundidas 1, 3, 7 y 14 días postrasplante. El tejido fue procesado para histología convencional y para la detección inmunohistoquímica del factor transformador del crecimiento-ß1. Resultados: La papila apical trasplantada desarrolló osteodentina. En fases tempranas postrasplante se observaron células parecidas a los odontoblastos que se organizaron en empalizada y segregaron dentina que se depositó sobre su superficie apical o secretora. Esta dentina evolucionó a osteodentina caracterizada por perder su estructura tubular e incluir a las células odontoblásticas en lagunas de su matriz. Finalmente, la osteodentina presentó procesos líticos mediados por células de tipo osteoclasto. Durante todo el proceso la expresión del factor transformador del crecimiento-ß1 se restringió a las células mesenquimales, a la matriz del trasplante y a las zonas circundantes del huésped, estando ausente en los odontoblastos, a diferencia de lo que sucede durante la odontogénesis normal. Conclusión: La diferente localización de la expresión del Factor Transformador de crecimiento ß1 entre el tejido hospedero y el trasplantado sugieren que en condiciones de trasplante heterotópico de papila dental la mediación inflamatoria del Factor Transformador de crecimiento beta1 prevalece sobre su papel morfogenético.

Palabras clave: *Odontoblasto, Papila dental, TGF-ß1, Trasplante.*

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