

## IMMUNOLocalIZATION OF THE TGFB1 SYSTEM IN SUBMANDIBULAR GLAND FIBROSIS AFTER EXPERIMENTAL PERIODONTITIS IN RATS

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### ABSTRACT

Saliva is the first barrier to entry of bacteria and viruses into the body. The submandibular glands (SMG) contribute to the maintenance of oral health and regulation of immune/inflammatory responses. Previous studies suggest that transforming growth factor beta 1 (TGFB1) may contribute to salivary gland fibrosis but the expression of the TGFB1 system in the SMG has not been elucidated. Thus, the aim of this study was to analyze in rat SMG the immunolocalization of TGFB1 and its specific receptors ALK5 (pro-fibrotic) and ALK1 (pro-proliferative) and the co-receptor endoglin (EDG) in a bilateral experimental periodontitis (EP) model (cotton thread ligature around the neck of the first lower molars) for 1 and 6 weeks. Fixed SMG were embedded in paraffin and serially cut for routine hematoxylin–eosin staining for histological analysis or immunohistochemical techniques by diaminobenzidine detection. SMG histology from animals with EP showed time-dependent structural changes involving marked reduction in the height of the contoured ducts, cell destruction, loss of secretory

granules, periductal congestion and excess connective tissue surrounding these ducts indicative of a fibrotic process, compared to control SMG. TGFB1, ALK5 and ALK1 receptors and the co-receptor EDG were mainly immunolocalized in ductal cells and in the fibrotic areas in EP groups. The expression of the pro-fibrotic ALK5 receptor was increased in areas of fibrosis in SMG of animals with EP. In SMG of rats with EP, the localization of the TGFB1 specific receptors in the ducts and cells from fibrotic areas, due to the expression of TGFB1 in the surrounding areas, might indicate paracrine and autocrine actions exerted by TGFB1 via its specific receptors. The results of this study suggest that TGFB1 promotes fibrosis, inducing cell proliferation via ALK1 and EDG receptors and stimulates fibrosis related-processes via ALK5 receptor, which could lead to abnormal secretor activity of the SMG during periodontal disease.

**Key words:** Submandibular gland, Transforming Growth Factor beta1, Activin Receptors, Fibrosis, Periodontitis.

## INMUNOLocalIZACIÓN DEL SISTEMA TGFB1 EN FIBROSIS DE LA GLÁNDULA SUBMANDIBULAR BAJO PERIODONTITIS EXPERIMENTAL EN RATAS

### RESUMEN

La saliva es la primera barrera para la entrada de bacterias y virus en el cuerpo. Las glándulas submandibulares (GSM) contribuyen al mantenimiento de la salud oral y a la regulación de las respuestas inmuno-inflamatorias. Estudios previos sugieren que el factor de crecimiento transformante beta 1 (TGFB1) puede contribuir a la fibrosis de las glándulas salivales, pero la expresión y localización del sistema TGFB1 en las GSM no ha sido dilucidada. El objetivo del presente trabajo fue analizar por inmuno-histoquímica en las GSM de ratas la expresión de TGFB1 y sus receptores específicos ALK5 (pro-fibrótico) y ALK1 (pro-proliferativo) y el co-receptor endoglina (EDG) en un modelo de periodontitis bilateral experimental (PE) (hilo de algodón alrededor del cuello de los primeros molares inferiores) durante 1 y 6 semanas. Las GSM fueron fijadas y embebidas en parafina para realizar cortes seriados los cuales se tiñeron con hematoxilina-eosina para analizar la histología o se procesaron para realizar la técnica de inmunohistoquímica mediante detección con diaminobenzidine. La histología de las GSM de animales con PE reveló cambios estructurales tiempo dependientes, con una marcada reducción de la altura de los conductos, destrucción celu-

lar, pérdida de gránulos secretores, congestión periductal y exceso de tejido conectivo que rodea los conductos, indicando un proceso de fibrosis respecto de las GSM de animales control. TGFB1, ALK5 y ALK1 y el co-receptor EDG fueron principalmente inmunolocalizados en las células que forman los ductos y en las áreas de fibrosis en los grupos con PE. La expresión del receptor pro-fibrótico ALK5 se incrementó en las áreas de fibrosis en GSM de animales con PE. En GSM de ratas con PE, la localización de los receptores específicos de TGFB1 en las células de los conductos y áreas de fibrosis, junto con la expresión de TGFB1 en las áreas circundantes, podría indicar acciones paracrinas y autocrinas ejercidas por TGFB1 a través de sus receptores específicos. Los resultados de este estudio sugieren que TGFB1 podría inducir un proceso de fibrosis promoviendo la proliferación celular a través de los receptores ALK1 y EDG, y favoreciendo procesos relacionados con la fibrosis a través de su receptor ALK5, lo que conduciría a una actividad secretora anormal de la GSM durante la enfermedad periodontal.

**Palabras clave:** Glándula submandibular, factor de crecimiento transformante beta1, receptores de activina, fibrosis, periodontitis.

## INTRODUCTION

Periodontal diseases have been well described and characterized according to different degrees of inflammation, eventually leading to the destruction of teeth-supporting tissue. Periodontitis is characterized by chronic activation of the immune response and synthesis of several cytokines and proteases. Cytokines induce alterations in the cellular metabolism of the connective tissue concomitant with the destruction of the periodontal tissue and tooth loss<sup>1</sup>.

The pair of submandibular glands (SMG) is involved in the maintenance of the oral health and regulation of immune/inflammatory responses<sup>2</sup>. The SMG has a ductal structure that opens into the oral cavity with secretory end pieces, the mucous and serous acini, producing saliva<sup>3</sup>. A time-dependent link has been observed between experimental periodontitis (EP) and SMG activity, mediated by systemic and neural mechanisms. Initially, the EP stimulates glandular secretion, while the progression of the disease affects the normal function of the gland, leading to histological and biochemical changes, including an inflammatory response and reduced secretion<sup>4,5</sup>.

There is growing evidence showing the relationship between cytokines and the pathological fibrotic process of different tissues, characterized by the development of excess fibrous connective tissue, as a consequence of a tissue repair process that triggers an increase in the production and deposition of extracellular matrix. Among these cytokines, the transforming growth factor beta 1 (TGF $\beta$ 1) plays an important role. The TGF $\beta$ 1 inhibitory and stimulatory properties have been described in the regulation of cellular homeostasis in both physiological and pathological stages<sup>6-9</sup>. The effects of TGF $\beta$ 1 are exerted via specific type I and type II serine/threonine kinase receptors. Type II receptor (TGF $\beta$ RII) transphosphorylates and activates type I receptor (TGF $\beta$ RI). Two TGF $\beta$ RI have been described: activin receptor-like kinase 1 (ALK1), which signals via phosphorylation of Smads 1/5, and activin receptor-like kinase 5 (ALK5), which signals via phosphorylation of Smads 2/3<sup>10</sup>. In line with this, endoglin (EDG) appears to be a key co-receptor that contributes to an efficient transduction pathway of ALK-1<sup>11</sup>. In this context, it has been observed that deregulation mechanisms of TGF $\beta$ 1 appear to be involved in the progression of diseases

such as cancer and autoimmune fibrosis<sup>6</sup>. However, the expression of the TGF $\beta$ 1 system in the SMG affected by periodontal disease is unknown. The aim of this study was to analyze the localization of TGF $\beta$ 1 and its specific receptors ALK5 and ALK1 and the co-receptor EDG in rat SMG after 1 and 6 weeks of EP induction.

## MATERIALS AND METHODS

### Animals

Adult male Wistar rats from our own colony with initial body weight 220-250 g were randomly divided into 4 groups (n=8 animals per treatment): (1) 1- week control, (2) 1- week bilateral EP, (3) 6-week control, (4) 6- week bilateral EP. They were kept in group cages in an animal room with a photoperiod of 12 h light (07.00–09.00 h), room temperature 22–25° C, humidity: 52–56% and fed standard Purina chow pellets and tap water *ad libitum*. All experiments were performed following the National Institute of Health guidelines for the care and use of laboratory animals (NIH 85-23, revised in 1985) and protocols were approved by the Ethical Commission of the School of Dentistry, University of Buenos Aires.

### Induction of experimental periodontitis

EP was performed as previously reported<sup>5</sup>. EP was induced under general anesthesia with a mixture of 2% xylazine hydrochloride (5 mg/kg; i.p.) and 5% ketamine hydrochloride (50 mg/kg; i.p.). A cotton thread ligature was placed around the neck of both first lower molars (bilateral EP) and served as a retention device for oral microorganisms. The ligature was pushed into the gingival sulcus and left in place until sacrifice (1- and 6 weeks post surgery). In the 6 week EP treatment, the thread was renewed every 2 weeks in order to prevent the ligature from coming out of the sulcus, thereby not meeting the desired objective. The SMG and submandibular lymph nodes were removed and weighed (mg), after which they were fixed in 4% PFA for histological and immunohistochemical studies.

### SMG histology and immunohistochemistry

The tissues mounted in paraffin were cut at 5- $\mu$ m sections, dewaxed in xylene and rehydrated in graded alcohols. For each specimen, at least 3 to 5 slides were stained with hematoxylin/eosin for

general histological inspection. Two specialists reviewed all the SMG samples. For immunohistochemical analysis, endogenous peroxidase activity was inhibited in tissue sections using 0.5% v/v H<sub>2</sub>O<sub>2</sub>/methanol. Sections were blocked for 1 h with 15% normal goat serum in phosphate-buffered saline (PBS) and then incubated overnight at 4°C with primary antibody (1:100 diluted rabbit anti-

TGFB1, sc-146; 1:100 diluted rabbit anti TGFB RI [V-22], sc-402; 1:100 diluted rabbit anti TGFB RI [T-19], sc-398; 1:100 diluted rabbit anti-endoglin, sc-20632, Santa Cruz Biotechnology, Inc., USA). After 3 rinses in PBS, sections were incubated for 1h at room temperature with the appropriate 1:200 diluted biotinylated secondary antibody (Vector Labs, UK). After further washing in PBS, sections were incubated with 1:100 diluted streptavidin–peroxidase complexes (ABC kit, Vector Labs, UK). Development of peroxidase activity was achieved with 0.05% w/v 3,3-diaminobenzidine and 0.1% v/v H<sub>2</sub>O<sub>2</sub> in Tris–HCl. Negative controls were processed simultaneously by omitting the primary antibody or pre-absorbing the primary antibody with specific synthetic peptides.

### Statistical Analysis

Mean and standard error (SEM) were calculated and the InfoStat Software (version 2011, developed by Statistics Department, National University of Córdoba) was used for calculating differences between 2 groups using Student's t test. A *p*-value of less than 0.05 was considered significant.

## RESULTS

### Histology of the SMG in rats with bilateral EP

The weight of the SMG and submandibular lymph nodes in the EP groups was significantly higher than control SMG in both 1- and 6- week treatments ( $p < 0.05$ ) (Fig. 1A). The histology of the SMG of animals with 1- and 6- week EP showed alterations of the epithelial architecture and ducts, partial loss of material granular secretion and periductal edema, as well as an excess in the connective tissue surrounding the ducts, indicative of a fibrotic process compared to the control SMG (Fig. 1B).

### Detection of TGFB1, ALK5, ALK1 and co-receptor EDG in SMG from rats with EP

TGFB1 and its specific receptors ALK1 and ALK5 and co-receptor EDG were immunolocalized in the cell cytoplasm of the ducts of control SMG and SMG from animals with 1- and 6 week EP (Fig. 2). TGFB1, ALK1 and EDG were localized in the apical zone and nearest to the lumen while ALK5 showed diffuse localization within cell cytoplasm (Fig. 2).

The expression of TGFB1 and its specific receptors was also predominantly detected in the cytoplasm

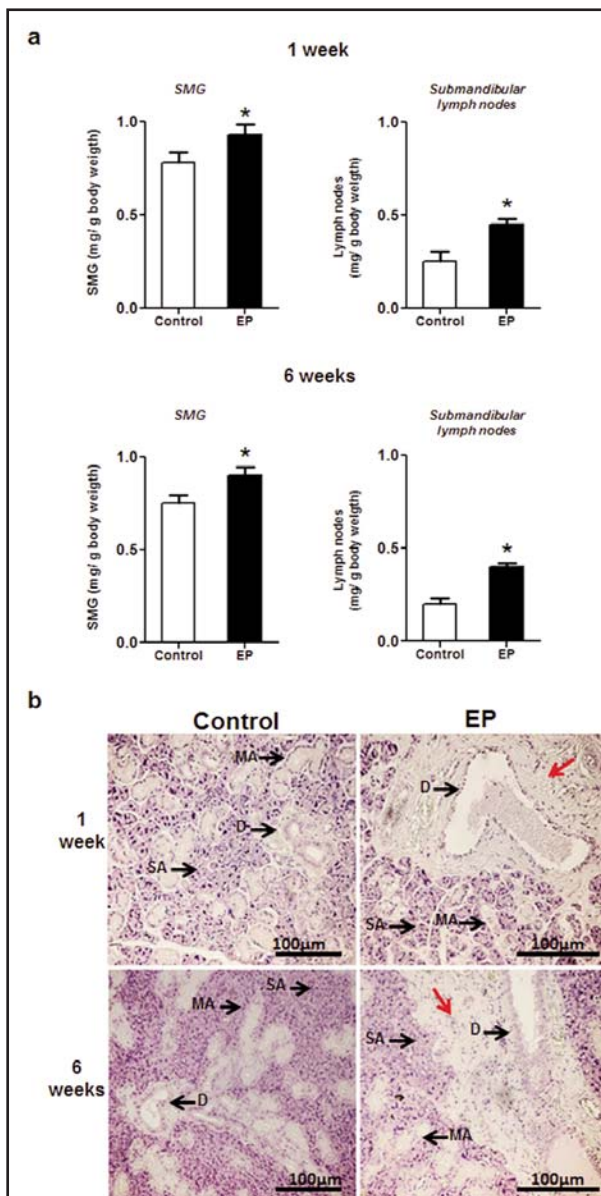


Fig. 1: Weight of SMG and submandibular lymph nodes (A) and general histology of SMG (B) in animals with 1- and 6-week EP. (B) Note the SMG of periodontitis groups with fibrotic areas surrounding ducts (red arrows). Data are plotted as mean  $\pm$  SEM ( $n = 6$ ). \* $p < 0.05$  different from control group. EP: experimental periodontitis; D: duct; MA: mucous acini; SA: serous acini.

of cells from the fibrosis areas surrounding the ducts in the affected SMG from animals with 1- and 6- week EP (Fig. 2). TGFB1 was also detected in mucous acinus (MA) and predominantly observed only in SMG of EP groups. Concerning the expression of ALK5, most of the cells from fibrosis areas were immunopositive for this receptor in animals with 6- week EP while the localization of ALK5 was detected in cell clusters in animals with 1-week EP (Fig. 2). Serous acinus (SA) showed no immunostaining for ALK5 and only a few immunopositive MA were observed in SMG from all treatment groups. The expression of ALK1 and its co-receptor EDG was mainly observed in cells of fibrosis areas with intense immunostaining in the affected SMG from animals with 1- and 6-week EP (Fig. 2). Some immunopositive MA for ALK1 and EDG was observed in the SMG from EP treatment groups.

## DISCUSSION

TGFB1, a pro-fibrotic cytokine, participates in diverse biological processes including inflammation, fibrosis, tissue regeneration and epithelial-mesenchymal transition<sup>6,12-15</sup>. In this study, we followed the expression of TGFB1 and its specific receptors ALK5 and ALK1 and the co-receptor EDG in normal SMG and in SMG after 1 and 6 weeks of induction of EP in adult rats. To our knowledge, this is the first report of global analysis of the expression of the TGFB1 system in SMG in EP conditions.

Normal SMG secretor activity is essential because saliva is the first barrier to entry of bacteria into the body, so changes in secretion are important in the onset and progression of oral infectious processes<sup>16</sup>.

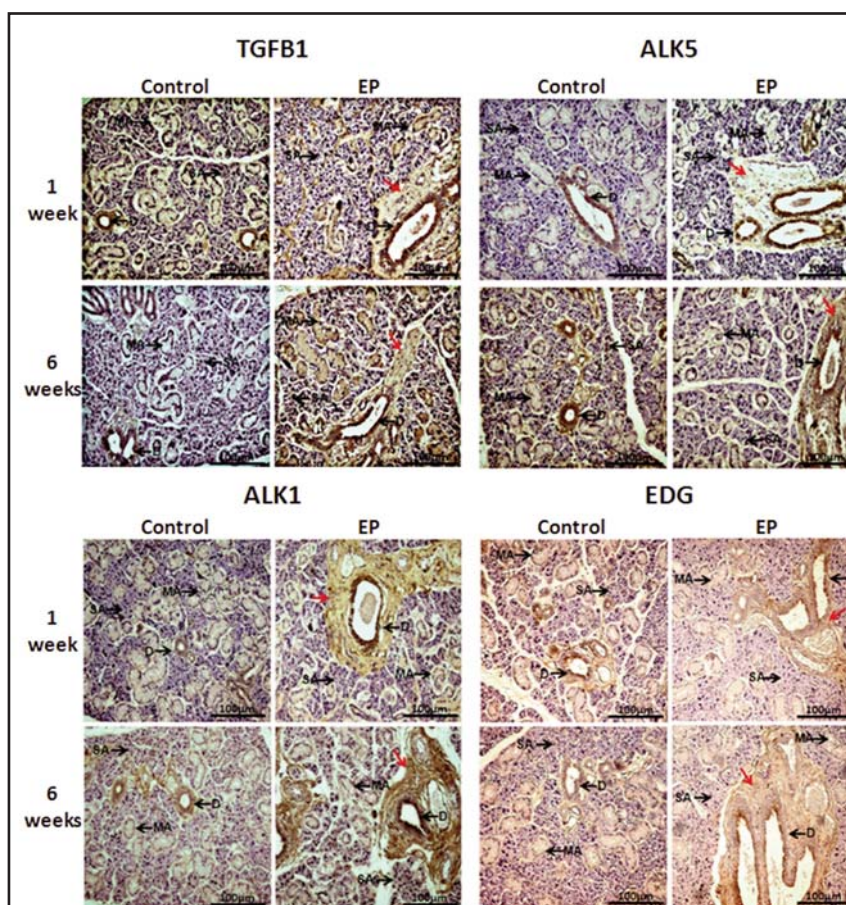


Fig. 2: Immunolocalization of the TGFB1 system in SMG from animals with 1- and 6-week EP. Note that fibrotic areas (red arrows) of the SMG of periodontitis groups are intensively immunostained with TGFB1. These areas were also immunopositive for ALK5 and ALK1 receptor and co-receptor EDG, particularly, in 6-week EP. EP: experimental periodontitis; D: duct; MA: mucous acini; SA: serous acini; EDG: endoglin.

Using a 1- and 6- week EP model, we observed histological changes in the SMG, mainly including the appearance of large fibrotic areas that could affect the normal secretor function of the gland. We detected alterations of the epithelial architecture and the ducts, partial loss of material granular secretion and excess in the connective tissue surrounding the ducts indicative of a fibrotic process in SMG after induction of EP. Moreover, SMG and lymph nodes weighed more in animals with EP than in control animals regardless of the duration of the treatment. This may be explained by the fact that EP produced severe structural changes involving a marked reduction in the height of the granular convoluted ducts, cell destruction, loss of secretory granules, increased fibrosis and periductal congestion. Under these experimental treatments, there is an excess of immunopositive cells for TGFB1 in the

connective tissue surrounding the ducts, indicative of a fibrotic process. In line with this, Hall *et al.*<sup>17</sup> have reported an increase in TGFB1 expression in salivary glands leading to hypofunction of the tissue due to the replacement of normal glandular parenchyma with interstitial fibrous tissue. We recently reported altered function of the SMG, including partial loss of secretor granular material with reduced salivary secretion and periductal oedema in EP treatments<sup>5</sup>.

Concerning the type of the TGFB1 receptor, it has been shown that the presence of the co-receptor EDG promotes TGFB1 signaling via ALK1 receptor leading to cell proliferation and attenuates the ALK5 signaling that leads to cell cycle arrest<sup>9,18</sup>. There is also growing evidence indicating that in pathological conditions, TGFB1 stimulates fibrosis through deposition of extracellular matrix and epithelial-mesenchymal transition via ALK5 receptor and activation of Smad2/3<sup>19-21</sup>. In the present study, the expression pattern of ALK1 and its co-receptor EDG was similar and remained constant in the fibrotic areas of SMG in all EP

treatments. This could indicate that TGFB1 induces a proliferative response in the connective tissue surrounding the ducts, stimulating the fibrotic process. In line with this, the quantity of cells in fibrotic areas expressing ALK5 increased in SMG concomitant with the days of the EP treatment, which may reflect a fibrotic process that becomes more aggressive with the duration of the periodontitis.

In conclusion, to our knowledge, this is the first report of the expression and localization of TGFB1 and its receptors ALK5 and ALK1 and co-receptor EDG in the SMG. We postulate that TGFB1 would be a key factor in SMG during periodontitis conditions, probably stimulating tissue damage, fibrosis and morphological alterations associated to abnormal secretor activity. The SMG of animals with EP appears to be an interesting model for studying diverse mechanisms that promote gland fibrosis exerted by TGFB1. Further insights into TGFB1 signaling in SMG could identify novel therapeutic targets for salivary gland fibrosis, whether or not associated to periodontal disease.

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