Immunohistochemical localization of tumor necrosis factor-alpha and interleukin-6-during orthodontic movement in rats

Milagres, D.[1], Rueff-Barroso, CR.[1], Bolognese, AM.[2], Costa, AMA.[3] and Porto, LC.[3]*

[1]Master of Science, Tissue Repair Laboratory, Department of Histology and Embriology, Rio de Janeiro State University

[2]Department of Orthodontics and Odontopediatrics, Rio de Janeiro Federal University

[3]Tissue Repair Laboratory, Department of Histology and Embriology, Rio de Janeiro State University, Av. Prof. Manuel de Abreu 444, 3º andar, CEP 20551-170, Rio de Janeiro, RJ, Brasil

*E-mail: lcporto@uerj.br, lcmporto@terra.com.br

Abstract

The tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are proinflammatory cytokines that have been associated to bone remodeling and bone deposition. However, their localization in the periodontal ligament (PDL) in vivo during the orthodontic movement is not well determined. TNF-α and IL-6 expression in PDL of maxillary rat incisors submitted to orthodontic movement was detected by immunohistochemistry at 6 and 12 hours and 4, 7, 21 and 28 days of force application. Alveolar bone lining cells expressed IL-6 in traction areas. In compression areas, some osteoblasts near osteoclasts expressed IL-6. TNF-α was essentially detected in osteoclasts, in compression areas, with 12 hours of force application. These cells expressed TNF-α and IL-6 in all time points studied. This study confirms that orthodontic force application induces TNF-α and IL-6 expression by PDL cells suggesting an essential role in bone–PDL remodeling.

Keywords: periodontal ligament, orthodontic, immunohistochemistry, TNF-α, IL-6.

1 Introduction

The osteoblasts and osteoclasts activities are modulated by hormones, cytokines and growth factors (HEYMANN and ROUSSELLE, 2000). Previous studies have implicated certain cytokines in bone remodeling in vitro and in vivo, including Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-6 (IL-6) (VOLEJNIKOVA, MARKS Jr. and GRAVES, 2002). TNF-α is one of the most potent osteoclastogenic cytokines produced in inflammation (ZOU, HAKIM, TSCHOEP et al., 2001). It activates nuclear factor-kappaB (NF-κB), enhances Monocyte-Colony Stimulating Factor (M-CSF) (KWAN, PADRINES, THEOLEYRE et al., 2004) or directly induces osteoclastogenesis (KUDO, FUJIKAWA, ITONAGA et al., 2002; LAM, TAKESHITA, BARKER et al., 2000) via Tumor Necrosis Factor Receptor I (ZOU, HAKIM, TSCHOEP et al., 2001). IL-6 is thought to be a major mediator of host defense against infection, and it regulates immune response in inflammed tissue. Proinflammatory cytokines, such as TNF-α, induce IL-6 production through the activation of the p38MAPK which, in turn, enhances activity of NF-κB (KUROKOUCHI, KAMBE, YASUKAWA et al., 1998). IL-6 mediates the stimulatory effects of TNF-α, IL-6 activates osteoclasts via RANKL or through an IL-6 receptor (PALMQVIST, PERSSON, CONAWAY et al., 2002) and induces osteoclastogenesis and bone (KOBAYASHI, TAKAHASHI, JIMI et al., 2000; KWAN, PADRINES, THEOLEYRE et al., 2004) and root resorption. TNF-α and IL-6 are of particular importance since they have been shown to be modulators of bone resorption (ALHASHIMI, FRITHIOF, BRUDVIK et al., 2001; UEMATSU, MOGI and DEGUCHI, 1996). The aim of this study was to localize TNF-α and IL-6 by immunohistochemistry on PDL during orthodontic movement in rats.

2 Material and methods

2.1 Animal treatment

Thirty male Wistar rats (*Rattus norvegicus albinus*), 3 months old, were divided into 6 groups with 6 and 12 hours and 4, 7, 21, and 28 days of orthodontic force application. In the control group, 3 animals had no orthodontic appliance.

The animals were anaesthetized with clorpromazine cloridrate (Amplictil, Rhodia Farma, São Paulo, SP, Brazil) and ketamine (Francotar, Virbac Laboratorios, Roseira, SP, Brazil), 25 mg/kg, IM. The orthodontic appliance consisted of a stainless steel round wire 0.016" (TP Orthodontics, São Paulo, SP, Brazil) with a double helix of 1.5 mm diameter at the median line, distant of the incisors approximately 7 mm back-forward and 1 mm far from the palate bone. The two segments of parallel wire circled the incisors by the distal surface and passed by the vestibular one. To activate the apparatus, it was used a dynamometer (Dentaurum 040-711, São Paulo, SP, Brazil) and measured (50 g/f) to tilted the teeth distally.

The Ethics Committee for Experimental Animals Use and Care (CEA) of IBRAG-UERJ approved all animal procedures and this study was in adherence with the COBEA (Brazilian College for Animal Experimentation) recommendations.

2.2 Tissue harvesting and processing

Animals were sacrificed and the anterior fraction of their maxillae were removed and fixed in 4% paraformaldehyde (Proquímios, Rio de Janeiro, RJ, Brazil), pH 7.4 for 48 hours and washed in current water for 2 hours. Dissected maxillae were demineralized in 10% EDTA (Proquímios, Rio de Janeiro, RJ, Brazil) pH 7.8 solution for 6 weeks, renewed at each 48 hours. Then, samples were washed in PBS pH 7.4 for 1 day and paraffin embedded. Sections of 5 μm thickness were obtained. They were analyzed and the histological aspects were recorded in the following areas: alveolar crest, named area 1, and 200, 1000 and 2000 apart from 1 in apical direction, respectively named area 2, 3 and 4 (Figure 1a). Serial sections were immunolabeled with anti-TNF-α and anti-IL-6 antibodies and the periodontal thickness measured in each mesial and distal areas.

2.3 Periodontal thickness evaluation

Three mesial and distal thicknesses were measured at random ($n \geq 3$) on four root areas in each experimental time. The values were expressed in μm, and data was analyzed using the Kruskal-Wallis Test to evaluate differences among the experimental time of each root area and the controls.

2.4 Immunohistochemical localization of TNF-α and IL-6

Immunodetection was performed using goat ABC Staining System (sc-2023, Santa Cruz Biotechnology, CA, USA). Sections were incubated for 5 minutes with PBS pH 7.2 at 37 °C and for 20 minutes with trypsin and $CaCl_2$ 0.1% pH 6.8 at 37 °C. After, rinsed in PBS pH 7.2 for 5 minutes and incubated with methanol solution of H_2O_2 3%, sections were washed thoroughly in distilled water and rinsed again. They were incubated with 1.5% normal blocking serum in PBS pH 7.2 for 1 hour at room temperature. Then they were incubated overnight at 4 °C with the primary antibody (goat anti-human TNF-α polyclonal antibody - sc-1350 or goat anti-mouse IL-6 polyclonal antibody - sc-1265, Santa Cruz Biotechnology, CA, USA), diluted 1:100 in 1.5% blocking serum in PBS pH 7.2. Negative control was incubated overnight with PBS pH 7.2. The sections were washed and incubated with 0.5% biotinylated second antibody in 1.5% blocking serum in PBS pH 7.2 for 2 hours at room temperature. Then washed, and incubated with 4% AB enzyme reagent in PBS pH 7.2 for 2 hours at room temperature, and washed again before incubated with peroxidase substrate. The sections were washed and counterstained in haematoxylin, dehydrated and mounted.

3 Results

3.1 IL-6 and TNF-α expression

For all experimental groups, there was no great difference among the 4 areas of the dental root. The bone crest alterations are quantitatively greater than those of the middle and the apical root, but not qualitatively, and at the apical root area in some animals the compression and the traction sides are reversed according the proximal orientation parameters.

In alveolar bone endosteum cells expressed IL-6 and TNF-α in traction area at all time points studied. Bone marrow osteoclasts, cortical osteocytes and cortical bone matrix did not express IL-6 or TNF-α in both traction and compression areas at all time points studied. Table 1 summarizes IL-6 and TNF-α immunodetection results and PDL histology. Cementum lining cells did not express IL-6 or TNF-α in any time points, neither in control nor in traction and compression areas. The alveolar bone lining cells express IL-6 at mesial side of control group (Figure 2a), these cells differentiate into osteoblasts and express IL-6 at the traction areas at all times of movement (Figures 2b-f). TNF-α expression by alveolar bone lining cells was not significant (data not shown).

At 6 and 12 hours of orthodontic movement, osteoblasts in traction area expressed IL-6 (Figure 2b), in compression area osteoclasts expressed IL-6 and TNF-α (data not shown). At 4 and 7 days of orthodontic movement osteoblasts in traction (Figures 2c and 2d) and compression areas expressed IL-6, and osteoclasts in compression area still express IL-6 and TNF-α (Figure 2g). At 21 and 28 days of orthodontic movement osteoblasts from traction (Figures 2e, f) and compression areas expressed IL-6, and osteoclasts from compression area expressed both IL-6 and TNF-α. At 21 days of orthodontic movement osteoclasts from traction area expressed TNF-α and IL-6, but at 28 days these cells were no more observed (data not shown).

Endothelial cells expressed IL-6 at all time points and regions studied. Edema was observed around vessels from 6 hours to 7 days of orthodontic movement, and during this period cells found around vessels expressed IL-6.

3.2 PDL thickness

Results concerning alterations in PDL thickness are presented in Figure 1b. In areas 1 and 2, traction and compression forces induced enlargement and diminution of PDL thickness, respectively. The enlargement in mesial face of the PDL was evident 6 hours and maximum values were found at 28 days in both areas. We found a biphasic response at the mesial face, the thickness median were close to the normal situation at 4 and 7 days for area 1 and 2. On the opposite face, compression resulted in the minor distance between cement and alveolar bone surface at days 4 and 7. In area 3, the mesial LPD showed less intense modification of its thickness, although significant decreasing of the thickness were observed in the distal face by 6 and 12 hours and at 7 days, medians close to the normal were obtained at 28 days. At the area 4, enlargement of both mesial and distal faces were observed.

4 Discussion

This work showed that IL-6 and TNF-α are expressed by the cells of the PDL during orthodontic movement. The incisors distal tipping caused bone resorption at compression areas and bone deposition at traction areas as previous described in literature (HELLER and NANDA, 1979). No root resorption was observed because of optimum force magnitude used. The PDL thickness confirmed the traction and compression areas since 6 hours of applied forces and was sustained with maximum measurements of the thickness at 28 days. However some variation in tissue characteristics can be found even among persons of a similar age (REITAN, 1967).

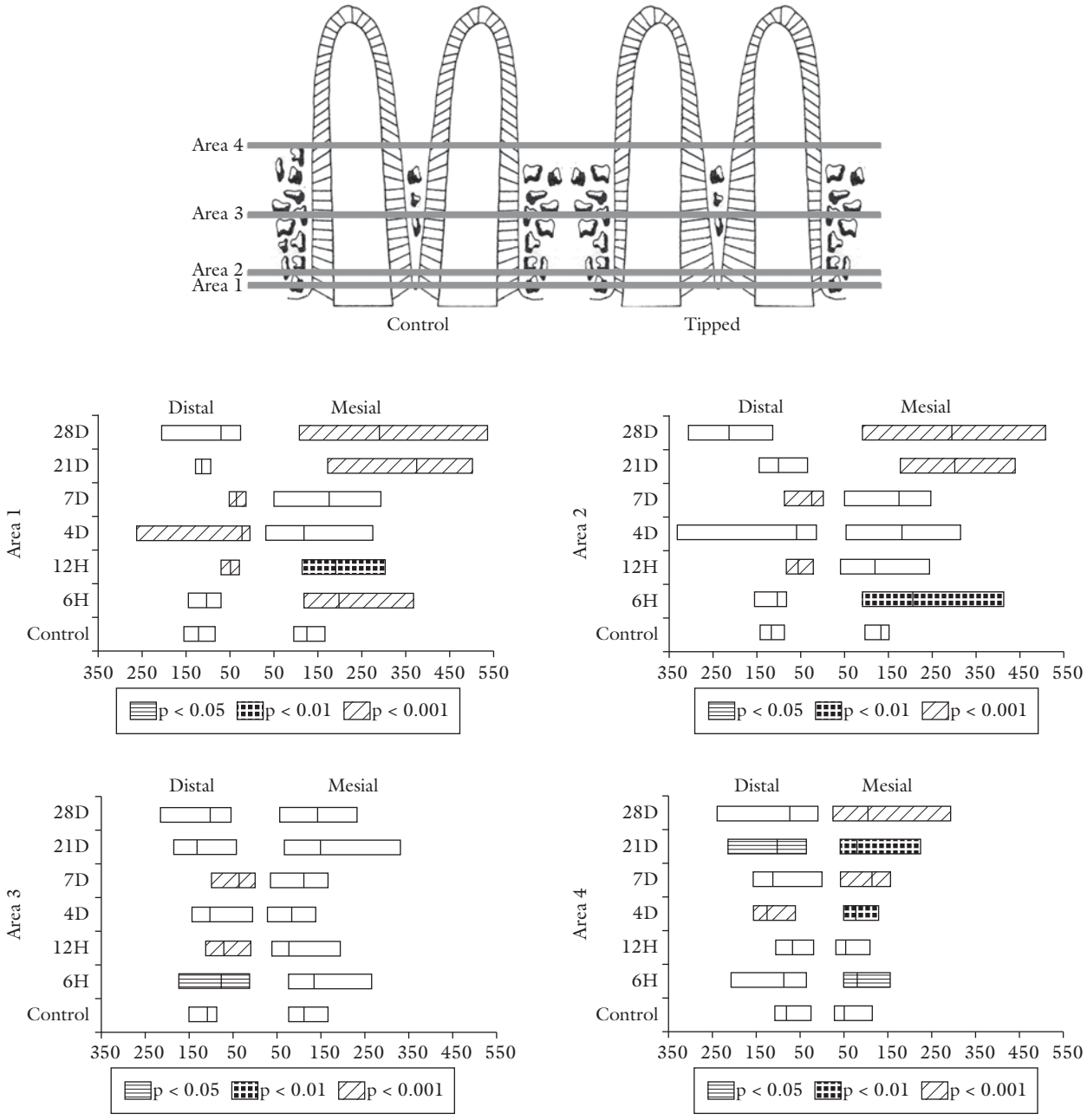

Figure 1. (A) The sectioned areas obtained from rat maxillae: alveolar crest, named area 1, and 200, 1000 and 2000 apart from 1 in apical direction, respectively named area 2, 3 and 4. (B) Periodontum thickness Bar indicates maximum, median and minimum values (μm) of the distance between alveolar bone and the external surface of the tooth at times studied. Kruskal-Wallis test.

The bone marrow cells expressed TNF-α and IL-6. Many in vitro studies have demonstrated the TNF-α expression by bone marrow stromal cells and its role on osteoclast differentiation and function (SUDA, TAKAHASHI, UDAGAWA et al., 1999; TAKAHASHI, TAKASHIBA, NAGAI et al., 1994). Detailed pathway analysis revealed that bone marrow cells have IL-6 receptors and activate signaling pathways through independent mechanisms (CHATTERJEE, STUHMER, HERRMANN et al., 2004).

The alveolar bone was irregular on periodontal surface at the compression areas after 12 hours of force application. Our findings are according with a study that evaluates the distributional changes of osteoclasts in periodontal tissues during orthodontic movement where many osteoclasts were observed in vascular canals of the alveolar bone crest near the pressure side of periodontal ligament after 6 hours of orthodontic force application (YOKOYA, SASAKI and SHIBASAKI, 1997). These cells were TNF-α and IL-6 positive. At this time, the number of osteoclasts was not increased in periodontal ligament. Yokoya, Sasaki and Shibasaki (1997) observed an increased number of osteoclasts at day 1 after tooth movement until day 7 in compression areas, they also observed some osteoclasts at traction side at the day 1 until day 14 and this number did not increased (YOKOYA,

Table 1. TNF-α and IL-6 immunohistochemistry and histological aspects of periodontal tissues submitted to orthodontic movement.

Time of movement	Traction area							Compression area						
	0	6 hours	12 hours	4 days	7 days	21 days	28 days	0	6 hours	12 hours	4 days	7 days	21 days	28 days
Periodontal ligament														
Alveolar área														
Aspect[A]	lin	lin	lin	irr	irr	irr	irr	lin	lin	irr	irr	irr	irr	irr
Alveolar bone lining cells	il6	(-)	(-)	(-)	(-)	(-)	(-)	neg	neg	neg	neg	neg	neg	neg
Osteoblasts	(-)	il6	il6	il6	il6	il6	il6	(-)	(-)	(-)	il6	il6	il6	il6
Osteoclasts	(-)	(-)	(-)	(-)	(-)	pos	(-)	(-)	pos	pos	pos	pos	pos	pos
Fibrous área														
Collagen fibers[C]	org	dis	dis	norg	norg	norg	org	org	com	com	norg	com	norg	org
Fibroblasts[F]	-	more	more	less	equal	equal	equal	-	equal	equal	less	equal	equal	more
Fibrocytes[F]	l	less	less	more	equal	equal	equal	-	equal	equal	more	equal	equal	less
Vascular áreas														
Edema[E]	A	P	P	P	P	A	A	A	A	A	A	A	A	A
Endothelial cells	il6	il6	il6	il6	il6	il6	il6	il6	il6	il6	il6	il6	il6	il6
Surrounding cells	neg	il6	il6	il6	il6	neg	neg	neg	neg	neg	neg	neg	neg	neg
Lymphomononuclear cells	(-)	pos	pos	pos	pos	pos	pos	(-)	(-)	(-)	(-)	(-)	(-)	(-)

IH: (-) - not found or not significant, pos - TNF-α and IL-6 positive, neg - negative. A: lin - linear, irr - irregular, C: org - organized, norg - disorganized. dis - disrupted and com - compressed; E: A - absent or P - present; F - equal, more or less cells subjectively compared to control.

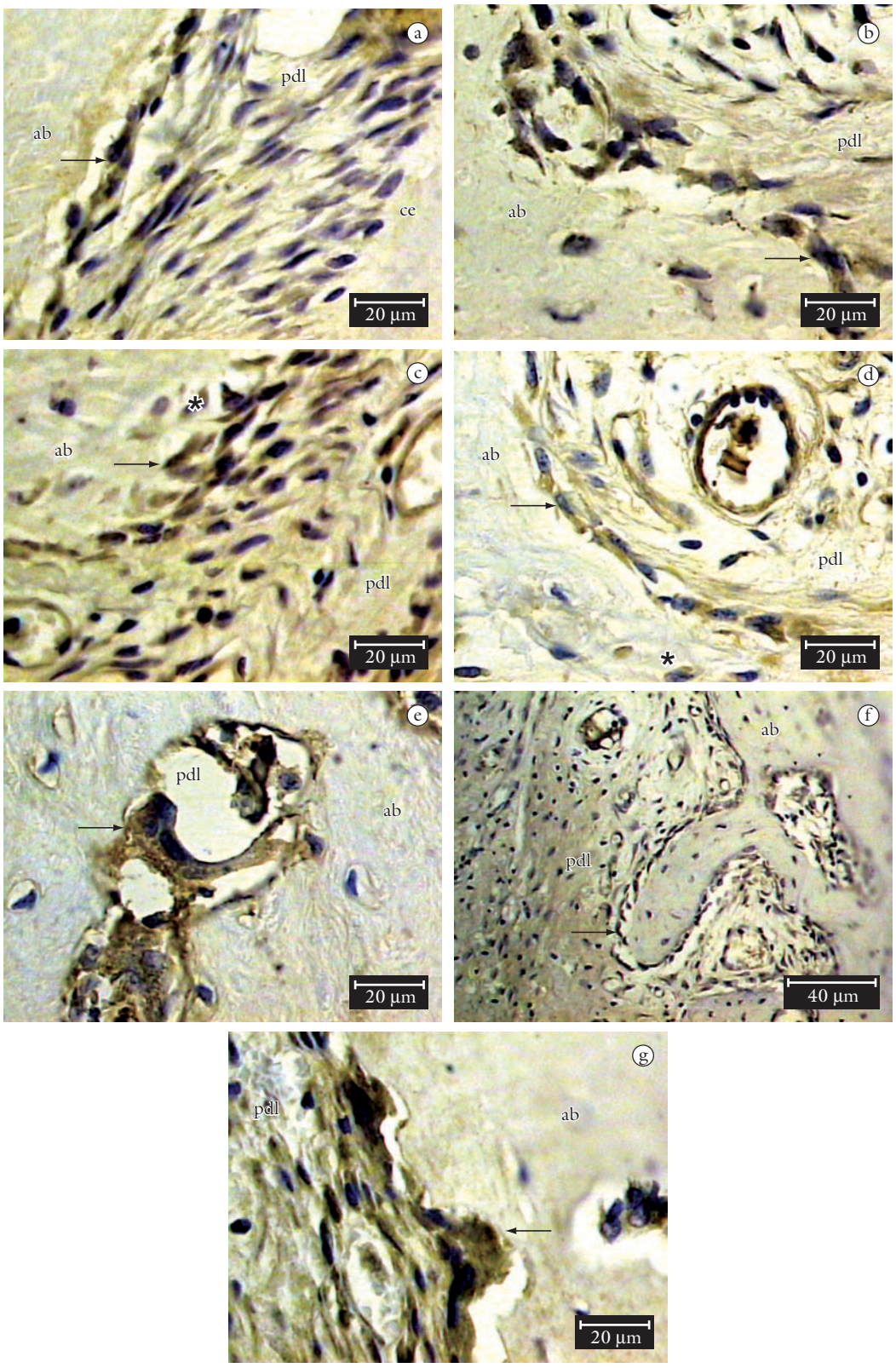

Figure 2. Immunohistochemistry for IL-6 and TNF-α. (A) Control group - mesial side. IL-6 positive alveolar bone lining cells (arrow). (B) Traction area at 6 hours of force application. IL-6 positive osteoblasts (arrow). (C) Traction area at 4 days of force application. IL-6 positive osteoblasts (arrow) recently incorporated to new bone matrix (asterisk). (D) Traction area at 7 days of force application. IL-6 positive osteoblasts (arrow) recently incorporated to new bone matrix (asterisk). (E) Traction area at 21 days of force application. IL-6 positive osteoblasts and osteoclasts (arrow). (F) Traction area at 28 days of force application. IL-6 positive osteoblasts (arrow). (G) TNF-α positive osteoclasts (arrow). ab – alveolar bone, ce – cementum, pdl – periodontal ligamentum.

SASAKI and SHIBASAKI, 1997). We observed some osteoclasts at periodontal ligament since 6 hours after force application in compression area. Alveolar bone with irregular periodontal surface a feature typical of bone deposition was observed since day 4 in traction areas, however we only observed osteoclasts at traction side at day 21.

Our findings on IL-6 expression showed that alveolar bone lining cells can express IL-6 even in absence of orthodontic force. These cells changed their shape differentiating into osteoblasts (ROBERTS and GOODWIN Jr., 1981) and continued expressing IL-6 at the traction areas during orthodontic movement. RANKL expression was detected on the PDL during orthodontic movement in both activated and non-activated osteoblasts (OSHIRO, SHIOTANI, SHIBASAKI et al., 2000) suggesting that these cells can express these cytokines in other biologic pathways.

On compression areas some osteoblasts expressed IL-6 near the osteoclasts that also expressed this cytokine. These findings agree with a previous study that showed increased levels of IL-6 mRNA on the compression areas in response to orthodontic tooth movement (ALHASHIMI, FRITHIOF, BRUDVIK et al., 2001). The same study didn't find TNF-α mRNA in response to orthodontic movement. The authors explain that no measurable mRNA was recorded to TNF-α in periodontal ligament probably because this cytokine is mainly released during the application of force and not produced again or the TNF-α mRNA is down regulated because the protein levels are still measurable in body fluids (ALHASHIMI, FRITHIOF, BRUDVIK et al., 2001). This could support the theory that the TNF-α is mainly released during the application of force and not produced again (ALHASHIMI, FRITHIOF, BRUDVIK et al., 2001). Moreover, Lowney and cols (LOWNEY, NORTON, SHAFER et al., 1995) using two different appliances, measured the amount of TNF-α at the sulcus at 5 minutes after the application of force and concluded that the mechanical system used did not alter TNF-α response although increased levels were detected after force application. It means that the quantitative response depends on the mechanical appliances. It is important to consider that the human gingival crevicular fluid is not a trusting way to measure the cytokines production by the PDL cells because many other cells of the periodontum can produce these cytokines induced by other stimulus (LOWNEY, NORTON, SHAFER et al., 1995). Our appliance promoted continuous force application and the immunohistochemistry showed direct intracellular TNF-α expression. This methodology is really effective to show the TNF-α expression in PDL during orthodontic movement. TNF-α was essentially expressed by the osteoclasts at the compression areas. These cells can be observed since 6 hours of orthodontic movement showing that bone resorption is already present in compression areas.

This work showed that the application of orthodontic force induces TNF-α and IL-6 expression by cells of periodontal ligament, reaffirming that the cytokines may play important roles in bone physiology control. The force and the functional appliances seems to influence directly the force-dependent TNF-α expression. However IL-6 PDL expression seems to be not only promoted by orthodontic force.

References

ALHASHIMI, N, FRITHIOF, L, BRUDVIK, P and BAKHIET, M. Orthodontic tooth movement and de novo synthesis of proinflammatory cytokines. *American Journal of Orthodontics and Dentofacial Orthopedics*, 2001, vol. 119, p. 307-312.

CHATTERJEE, M, STUHMER, T, HERRMANN, P, BOMMERT, K, DORKEN, B and BARGOU RC. Combined disruption of both the MEK/ERK and the IL-6R/STAT3 pathways is required to induce apoptosis of multiple myeloma cells in the presence of bone marrow stromal cells. *Blood*, 2004, vol. 104, p. 3712-3721.

HELLER, IJ and NANDA, R. Effect of metabolic alteration of periodontal fibers on orthodontic tooth movement. An experimental study. *American Journal of Orthodontics*, 1979, vol. 75, p. 239-258.

HEYMANN, D and ROUSSELLE, AV. Gp130 Cytokine family and bone cells. *Cytokine*, 2000, vol. 12, p. 1455-1468.

KOBAYASHI, K, TAKAHASHI, N, JIMI, E, UDAGAWA, N, TAKAMI, M, KOTAKE, S, NAKAGAWA, N et al. Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction. *The Journal of Experimental Medicine*, 2000, vol. 191, p. 275-286.

KUDO, O, FUJIKAWA, Y, ITONAGA, I, SABOKBAR, A, TORISU, T and ATHANASOU, NA. Proinflammatory cytokine (tnfalpha/IL-1alpha) induction of human osteoclast formation. *The Journal of Patology*, 2002, vol. 198, p. 220-227.

KUROKOUCHI, K, KAMBE, F, YASUKAWA, K, IZUMI, R, ISHIGURO, N, IWATA, H and SEO, H. TNF-alpha increases expression of IL-6 and ICAM-1 genes through activation of NF-kappab in osteoblast-like ROS17/2.8 cells. *Journal of Bone and Mineral Research*, 1998, vol. 13, p. 1290-1299.

KWAN, TS, PADRINES, M, THEOLEYRE, S, HEYMANN, D and FORTUN, Y. IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. *Cytokine Growth Factor Reviews,* 2004, vol. 15, p. 49-60.

LAM, J, TAKESHITA, S, BARKER, JE, KANAGAWA, O, ROSS, FP and TEITELBAUM, SL. TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. *The Journal of clinical investigation*, 2000, vol. 106, p. 1481-1488.

LOWNEY, JJ, NORTON, LA, SHAFER, DM and ROSSOMANDO, EF. Orthodontic forces increase tumor necrosis factor alpha in the human gingival sulcus. *American Journal of Orthodontics and Dentofacial Orthopedics*, 1995, vol. 108, p. 519-524.

OSHIRO, T, SHIOTANI, A, SHIBASAKI, Y and SASAKI T. Osteoclast induction in periodontal tissue during experimental movement of incisors in osteoprotegerin-deficient mice. *The Anatomical Record,* 2000, vol. 266, no. 4, p. 218-225.

PALMQVIST, P, PERSSON, E, CONAWAY, HH and LERNER, UH. IL-6, leukemia inhibitory factor, and oncostatin M stimulate bone resorption and regulate the expression of receptor activator of NF-kappa B ligand, osteoprotegerin, and receptor activator of NF-kappa B in mouse calvariae. *Journal of Immunology,* 2002, vol. 169, p. 3353-3362.

REITAN, K. Clinical and histologic observations on tooth movement during and after orthodontic treatment. *American Journal of Orthodontics*, 1967, vol. 53, p. 721-745.

ROBERTS, WE and GOODWIN Jr., WC. Heiner SR. Cellular response to orthodontic force. *Dental clinics of North America*, 1981, vol. 25, p. 3-17.

SUDA, T, TAKAHASHI, N, UDAGAWA, N, JIMI, E, GILLESPIE, MT and MARTIN, TJ. Modulation of osteoclast differentiation and

function by the new members of the tumor necrosis factor receptor and ligand families. *Endocrine Reviews*, 1999, vol. 20, p. 345-357.

TAKAHASHI, K, TAKASHIBA, S, NAGAI, A, TAKIGAWA, M, MYOUKAI, F, KURIHARA, H, MURAYAMA, Y. Assessment of interleukin-6 in the pathogenesis of periodontal disease. *Journal of Periodontology*, 1994, vol. 65, p. 147-153.

UEMATSU, S, MOGI, M and DEGUCHI, T. Interleukin (IL)-1 beta, IL-6, tumor necrosis factor-alpha, epidermal growth factor, and beta 2-microglobulin levels are elevated in gingival crevicular fluid during human orthodontic tooth movement. *Journal of Dental Research*, 1996, vol. 75, p. 562-567.

VOLEJNIKOVA, S, MARKS Jr, SC and GRAVES, DT. Tumor necrosis factor modulates apoptosis of monocytes in areas of developmentally regulated bone remodeling. *Journal of Bone and Mineral Research*, 2002, vol. 17, p. 991-997.

YOKOYA, K, SASAKI, T and SHIBASAKI, Y. Distributional changes of osteoclasts and pre-osteoclastic cells in periodontal tissues during experimental tooth movement as revealed by quantitative immunohistochemistry of H(+)-atpase. *Journal of Dental Research*, 1997, vol. 76, p. 580-587.

ZOU, W, HAKIM, I, TSCHOEP, K, ENDRES, S and BAR-SHAVIT, Z. Tumor necrosis factor-alpha mediates RANK ligand stimulation of osteoclast differentiation by an autocrine mechanism. *Journal of Cellular Biochemistry*, 2001, vol. 83, p.70-83.

Received May 8, 2008
Accepted April 21, 2009