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Full Length Research Paper

In vivo extracellular matrix protein expression by human periodontal ligament after stimulation with orthodontic force

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It is well known that the orthodontic force applied to teeth generates a series of events that remodel the periodontal ligament (PDL). Extracellular matrix proteins (ECM) are described as molecular regulators of these events. However, the exact contribution of these proteins in human PDL modeling by orthodontic force application *in vivo* is not known. The aim of this present study was to evaluate the protein expression of fibronectin, laminin and vitronectin by human PDL from teeth on which orthodontic force was applied. Twenty healthy individuals were included in the study. PDL was obtained from teeth after a 3-week treatment with orthodontic force. PDL-protein samples were separated on 7.5% SDS-PAGE Western blot analysis with specific monoclonal antibodies for fibronectin, laminin and vitronectin. Bands were visualized with an enhanced chemiluminescence detection system and densitometric. Scanning of bands was carried out to compare differences in protein expression. A significant increment in fibronectin (13.9%), laminin (16.5%) and vitronectin (14.2%) expression was found in PDL from teeth treated with orthodontic force for 3 weeks in comparison with teeth in the control group. Our results support the concept that molecular changes take place by application of orthodontic forces to the PDL. Over expression of these proteins suggests that extracellular matrix (ECM) remodeling could be generated in response to mechanical stress.

Key words: Extracellular matrix proteins, periodontal ligament, orthodontic force.

INTRODUCTION

The aim of orthodontic treatment is to relocate teeth positioned abnormally in the jaws. This is achieved by the application of continuous force (orthodontic force) on the tooth until this structure reaches a correct position in the jaw. The tooth is sensitive to the existence of pressures applied and consequently prepares a response mediated through bone, gingiva and the periodontal ligament (PDL). It is a fact that the physiologic response of bone during relocation of teeth with the application of orthodontic forces is regulated and mediated by the PDL. This structure is composed of mainly fibroblast cells. These cells play a very important role in remodeling the bone through activation of molecular events such as elevation of intracellular calcium (Nakago-Matsuo et al., 1996), changes in nitric oxide concentration (Nakago-Matsuo et al., 2000), prostaglandin type E2 (van der Pauw et al., 2000) and extracellular matrix (ECM) proteins (Matsuura et al., 1995).

ECM has been traditionally considered to be a structurally stable material that provides support for cells

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and tissues. At present, it is known that ECM is a complex ordered aggregate comprising a number of different macromolecules whose structural integrity and functional composition are important in maintaining normal tissue architecture, development and tissue-specific function (Labat-Robert et al., 1990; Simon 2004; Slater 1996; Orend and Chiquet-Ehrismann 2000). It has been demonstrated that ECM is capable of regulating cell growth, cell proliferation and differentiation, development, metabolic responses, cell shape, cell adhesion and migration (Ekblom, 1995; Carey, 1991; Sage, 2001; Coats and Faxon, 1997; Chiquet-Ehrismann, 1991). In conjunction with these studies has come the recognition of the importance of dysfunctional matrix components and abnormalities in ECM biosynthesis and catabolism, which have been related with inherited and acquired diseases or with normal or pathologic wound healing (Labat-Robert et al., 1990). Thus, it is recognized that ECM is very important in odontology because teeth are united to bone by means of the PDL (Anastasi et al., 2008; Kim et al., 2010; Kinumatsu et al., 2009).

PDL-ECM proteins play an important role in normal maintenance, repair and regeneration of both the PDL and adjacent hard tissues (Anastasi et al., 2008; Atsuta et al., 2005). Additionally, it is known that ECM responds to mechanical stimulation. Thus, ECM proteins play a central role in mediating the osseous remodeling that underlies physiologic and orthodontic tooth movement (Howard et al., 1998). However, the type of ECM proteins synthesized by PDL cells *in vivo* remains unknown to date.

Redlich et al. (2004) demonstrated that in cultured human PDL fibroblasts, application of external pressure induces increase of tropoelastin levels. Also, Howard et al. (1998) showed that PDL fibroblasts exposed to biaxial deformation exhibited an increase in both type I collagen and fibronectin and a decrease in tropoelastin expression. In addition, it has been demonstrated that an increase in tenascin expression takes place in primary culture of human periodontal cells after application of gravitational force (Theilig et al., 2001). Bearing in mind all these data, it is clear that studies carried out *in vitro* demonstrate that application of an external stimulus could have a direct effect on ECM proteins, particularly on the components of PDL.

Anastasi et al. (2008) demonstrated changes in the expression of collagen and fibronectin in the periodontal ligament on application of orthodontic forces for short (72 hours) periods through immunohistochemical, histological and electron-microscopic study. Likewise, the authors suggested that fibronectin facilitates the reorganization of the connective tissues during tooth movement in rats (Kim et al., 2010). A previous study demonstrated that a laminin-like molecule produced by periodontal-ligament fibroblasts (PLFs) induces gingival epithelial cell chemotaxis (Ohshima et al., 2006). On the other hand, it is suggested that the laminin in the connective tissue may induce epithelial cell migration, may provoke cell adhesion and migration of cells facing the tooth on the enamel surface of the regenerating junctional epithelium. (Masaoka et al., 2009). On the other hand, vitronectin is involved in the early stages of periodontal repair, and periodontal regeneration is achieved through formation of periodontal tissues that are composed of different matrix components specific to different types of periodontal tissues (Matsuura et al., 1995).

Periodontal ligament (PDL) cells possess osteogenic potential and play important roles in bone biology, such as necrotic processes, bone resorption, bone modeling, dental movement, osteoblast differentiation and inflammatory processes. These processes are measured by integrins by means of the extracellular signal-regulated kinase (ERK) pathway, as well as the production of extracellular-matrix proteins, such as the extracellular matrix; such as vitronectin, laminin and fibronectin. Several of the models that are achieved through the application of orthodontic mechanics were scaled to produce typical human stress levels in order to generate dental movement (Pavlidis et al., 2009; Viecilli et al., 2009; Kook et al., 2009; Anastasi, et al., 2008).

To establish the possible interrelationship *in vivo* between human PDL from teeth subjected to orthodontic forces and induction of EMC proteins, the present study was done. The purpose of the present study was to evaluate *in vivo* direct effect of orthodontic force on laminin, fibronectin and vitronectin expression present in human PDL.

MATERIALS AND METHODS

Reagents and antibodies

All electrophoresis reagents, molecular weight standards, Fuji x-ray films and nitrocellulose membrane were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Monoclonal mouse IgG_1 antibodies, anti-human fibronectin, anti-human vitronectin, and anti-human laminin were obtained from Chemicon International, Inc. (Temeluca, CA, USA). Compatible secondary antibodies for Western blotting conjugated with horseradish peroxidase and Western blotting chemiluminescence Luminol reagent were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and were used according to the instruction included by the manufacturer. All other chemical reagents were obtained from Merck (Merck de México, S.A.) of the best available quality.

Patient selection and application of orthodontic force

The study protocol was approved by the Joint Research and Ethics Committee of Instituto de Seguridad y Servicio Social de los Trabajadores del Estado (ISSSTE) Centro Medico Nacional “20 de Noviembre” in Mexico City. Informed consent forms were signed by subjects, all of whom were systemically healthy.

To carry out this study, 20 healthy adult patients (Table 1) of both sexes (50% male and 50% female) were recruited. These patients

were of legal age (18 years) and the patients were volunteers who went to the UNAM's orthodontics clinic of the postgraduate and research division, with the objective of having orthodontic treatment, that is, all of the volunteers had a dental disorder: dental crowding. Treatment contemplated the extraction of maxillary first premolars according to Proffit (2001). A fixed, prescribed appliance (0.018-inch slot Roth with a 0.016-inch nickel-titanium arch) was placed on each subject. The appliance was set in all teeth of both upper and lower arches with the exception of the maxillary first left premolar, which was considered the control tooth; the maxillary first right premolar was considered the experimental tooth. The latter was subjected to orthodontic force. A bracket with an angle of 20° with regard to the longitudinal axis and to the arch employed was placed on the right first premolar. This bracket was tied to the arch with an elestomeric module (GAC International, Inc., New York USA) and the tooth was subjected to orthodontic force of approximately 60 *g* (Dóntrix-Richmond; 3M, Brasil), during 3 consecutive weeks. After this time was completed and during which the brackets were left in place to act as the appliance's orthodontic strength, upper premolars were extracted which included right (study) and left (control) ones.

Periodontal ligament isolation

As soon as the tooth was extracted, we proceeded to remove entirely the PDL united to the tooth by means of curettage with a scalpel blade. Tissue thus obtained was then quickly placed on ice and washed with ice-cold PBS until it was bloodless. PDL was weighed and immediately homogenized in Ultra-turrax apparatus (Ultra-turrax T8 homogenizer, Fisher Scientific Pittsburgh, PA, USA) with Tris-buffer (0.01 M Tris-HCl pH 7.4; 0.255 M sucrose and 0.3 mM EGTA; 1% Nonidet P-40; 1 mM Na_3VO_4; 1 mM phenylmethylsulfonyl fluoride (PMSF); 0.05% (w/v) aprotinin). The homogenized samples were frozen until its subsequent use. Total protein concentration was determined by Lowry method (Lowry et al., 1951), using bovine serum albumin (BSA) as standard.

Western blot analysis

Laemmli (1970) sample buffer (Tris 0.125 M; SDS 4%; glycerol 10%; β-mercaptoethanol 4%; bromophenol blue 0.02%) was added to the homogenized samples and heated at 95°C for 4 min. A volume that contained identical amounts of total protein (100 µg) from PDL of control and experimental teeth was loaded onto 7.5% gel SDS-PAGE for separation by electrophoresis according to Laemmli (1970). After electrophoresis, protein was electrotransferred to nitrocellulose membranes for immunoblotting (Sugiyama et al., 2003). Membranes were blocked with 5% (w/v) low-fat powdered milk in Tris-buffered-saline (TBS) and subsequently were tested for separation of the following primary mouse monoclonal (IgG_1) antibodies (1:100): anti-human fibronectin, anti-human vitronectin and anti-human laminin. Protein bands were visualized using horseradish peroxidase (HRP)-conjugated secondary antibody and the enhanced chemiluminescence detection system used according to manufacturer's instructions (Santa Cruz Biotechnology Inc, CA USA). As control of the technique, we employed β-actin, according to that reported by Nishimura et al. (1996).

Data collection and statistical analysis

Data were collected from twenty independent experiments for each evaluation. The immunoblots films were scanned on a densitometer (Ultrascan Laser densitometer, Pharmacia LKB, Piscataway, NJ, USA) to quantify bands density. Results were expressed as a mean of optical density (OD) units ± standard error mean (SEM). Statistical analysis of the date was carried out with Mann-Whitney U test. *P*-values smaller than or equal to 0.05 were considered significant.

RESULTS

Characteristic of the population and recovered periodontal ligament of the teeth

The general characteristics of the population from whom teeth were obtained for this study are shown in Table 1. Fifty percent of the population was women on average years of age 19.6 ± 2.41 (18-24 years). Men were on average 22.6 ± 3.67 years of age (18-26 years). There were no statistical differences in age between groups of study.

The amount of PDL-tissue obtained from both groups was similar (135.34 ± 11.04 mg and 130.21 ± 10.0 mg for control and treated groups, respectively). No statistical significant differences in total protein between obtained periodontal ligaments of control teeth and those subjected to orthodontic force was found (0.815 ± 0.011 and 0.829 ± 0.014 mg/mg wet weight; control and treated group, respectively Table 1).

Western blot Analysis

Figure 1 shows a representative image of a Western blot done to detect fibronectin (1A), laminin (1B) and vitronectin (1C) protein expression in control teeth and in teeth treated with orthodontic force. Spots in the left panel show PDL obtained from control teeth, while spots in the right panel depict PDL obtained from teeth with 3-week orthodontic force treatment.

In addition, the result of densitometric scan to quantify intensity of protein bands for each protein was observed (beside the corresponding Western blot image). The previous graph illustrates the average (± SEM, $n = 20$) of densitometric analyses of sample stains corresponding to control group and samples of periodontal ligaments treated with orthodontic force for 3 weeks. Western blot analysis by densitometric scan showed an increase in the intensity of the stain in PDL samples from teeth with 3-week orthodontic force treatment.

An increase of 13.9, 16.5, and 14.2% was found in the expression of fibronectin (Figure 1A), laminin (Figure 1B) and vitronectin (Figure 1C), respectively, in comparison with their controls, with all cases been statistically significant ($p < 0.05$).

Since in the study, women and men were recruited, a comparative analysis of Western blots between both groups related to fibronectin, laminin and vitronectin expression in order to determine whether some

Table 1. General characteristic of the population included in the study and general periodontal ligament characteristic of the teeth

Population characteristic	**Values**	
Total number of subjects	20	
Number of women	10	
Age (mean + S.D.) of years	19.6 ± 2.41	
Range of age	18-24	
Number of men	10	
Age (mean + S.D.) of years	22.6 ± 3.67	
Age range (years)	18-26	
Periodontal ligament characteristic	**Wet weight (mg)**	**Total protein**
Left tooth (control)	135.34 ± 11.04	0.815 ± 0.011
Right tooth (experimental)	130.21 ± 10.00*	0.829 ± 0.014*

Characteristic of the population included in the study and general characteristics of the periodontal ligament obtained from the left teeth (control) and the right teeth (experimental) after the application of orthodontic forces by three weeks. Wet and total protein weights are expressed as mean± SEM
* No statistical difference (experimental and control) was found.

difference would exist was carried out. On the other hand, no statistical differences between treated or untreated-groups were found (data not show).

DISCUSSION

The present study shows that orthodontic force applied to teeth for 3 weeks resulted in an increase in the expression of laminin, fibronectin and vitronectin from human PDL. The changes found in the expression of these proteins can be interpreted as the response from PDL to the application of orthodontic force. These changes can be part of the general biochemical response that these proteins carry out where PDL—and consequently ECM-component—remodeling exist. It is a fact that PDL is a specialized form of ECM possessed by the tooth. Molecules that are composed of PDL exert an influence on biochemical processes that take place when a tooth is remodeled by the application of orthodontic force. Similarly, ECM components can regulate their functions in response to application of orthodontic force.

Previous *in vitro* studies demonstrated that application of external stimuli could have a direct effect on proteins comprised of ECM (Howard et al., 1998; Redlich et al., 2004; Theilig et al., 2001). Our results showed, probably for the first time that human teeth stimulated with orthodontic force present an increment in expression of the early-repair proteins of ECM that belong to PDL. Our experiments were managed *in vivo* after 3 weeks. We consider this period a sufficient time to find modifications in protein expression during long-term treatment. In support of this previously mentioned consideration, Sugiyama et al. (2003) reported alterations in lysosomal cystein protease levels in human teeth treated with orthodontic force for up to 7 days.

Orthodontic force induces biochemical responses in PDL, but ECM-dependent molecular mechanisms in orthodontically induced periodontal remodeling remain unclear to date. Previous studies *in vitro* and *in vivo* indicated that mechanical stress induces changes in ECM molecular components of teeth (Anastasi et al., 2008; Kim et al., 2010; Kinumatsu et al., 2009; Howard et al., 1998; Redlinch et al., 2004; Theilig et al., 2001). Mechanical stress is detected by fibroblasts, the most common cell type in PDL. Fibroblasts modify ECM proteins and result in alterations in tooth adhesion while mechanical stress was present (Sugiyama et al., 2003). In this way, PDL plays important roles in the function and regeneration of the tooth support apparatus (Schuppan and Ruhl 1994; Schuppan et al., 1994; Anastasi et al., 2008). Fibronectin, laminin and vitronectin are important components of PDL (Steffensen et al., 1992); these proteins could participate in specialized functions such as tooth remodeling.

Analysis of our results obtained in *in vivo* conditions showed an important increase in fibronectin, laminin and vitronectin expression due to application of orthodontic force. Our data strongly suggest that these proteins can participate in ECM organization during the remodeling process of teeth induced by mechanical stress. This is not surprising because these proteins are involved in complex molecular processes such as growth and differentiation of cells during wound healing, adhesion and migration (Kinumatsu et al., 2009), cell attachment (Lallier et al., 2001), and in processes of ECM organization related with cellular adhesion (Orend and Chiquet-Ehrismann, 2000; Carey, 1991). It is well known that cell adhesion molecules are important in the behavior of cells, such as cell shape, differentiation, migration, intracellular signaling, etc (Orend and Chiquet-Ehrismann, 2000).

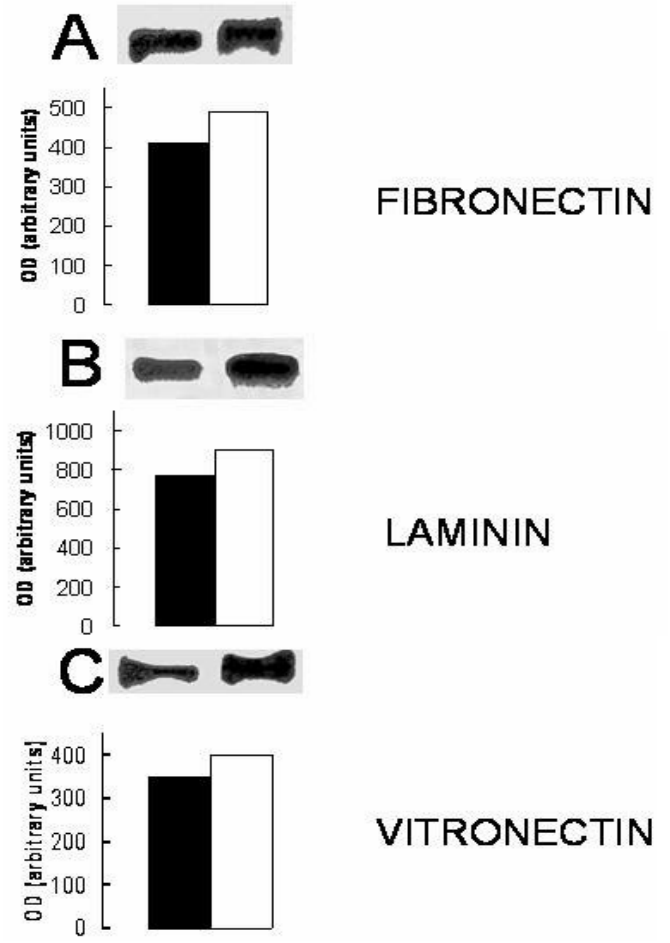

Figure 1. Western blot analysis of fibronectin, laminin and vitronectin expression that are in the periodontal ligament of teeth after 3 weeks of orthodontic forces (stain on right side) or that proceed from control teeth (stain on left side). A volume that contained identical amounts of protein (100 µg) from periodontal ligament of control and experimental subjects was loaded from each sample onto 7.5% gel SDS-PAGE. Protein were transferred onto nitrocellulose membranes, probed with the corresponding antibodies against fibronectin laminin and vitronectin and visualized with the enhanced chemiluminescence detection system to reveal proteins (A, B, and C, respectively). Densitometric scanning of bands to compare intensity of signals for the different proteins is beside the Western blot. Band intensities are expressed as optic density (OD) units. $P < 0.05$

Some information is currently available concerning the biological significance of cell adhesion molecules associated with PDL. Laminin is found in basement membranes, where it mediates epithelial cell attachment to type IV collagen (Terranova et al., 1980; Kariya and Miyazaki, 2004). Our results show that laminin had the highest expression when the tooth was stimulated with orthodontic force (an increment of up to 16.5% with regards to control) (Figure 1B). This indicates that laminin may be an important modulator in the repair processes that continuously take place in the PDL of teeth with mechanical stress.

Fibronectin is involved in extracellular matrix organization and adhesive interaction of cells (Yamada, 1983). Fibronectin was found widely distributed in baboon peridontium. Connective tissue matrix of marginal gingiva, endosteal spaces, as well as PDL showed the presence of fibronectin (Steffensen et al., 1992). In presence of tissue inflammation in dog, fibronectin was localized to an amorphous material interpreted as partially degraded fibers (Cho et al., 1984). On the other hand, vitronectin has been found associated with elastic fibers of loose connective tissues and was also found to be increased in fibrotic tissues that included myelofibrotic bone marrow and sclerotic glomeruli. These findings have suggested a role for vitronectin in the inflammation process and repair (Dahlback et al., 1986; Reilly and Nash, 1988).

With all of the previously mentioned material in mind, we hypothesized that laminin, fibronectin and vitronectin are proteins that play a role in the inflammatory process that takes place when orthodontic force is applied to teeth. In addition, it appears that increase in laminin, fibronectina and vitronectin expression is part of the remodeling process in teeth during mechanical stress related with orthodontic force. Further investigations are warranted with respect to the role of fibronectin, laminin and vitronectin in periodontal disease pathogenesis and regenerative processes.

Previous reports (Anastasi et al., 2008) demonstrate that changes in proteins of the periodontal ligament's extracellular matrix could be determined by variations of periodontal-ligament scaffold geometry, in that the movements of contraction and relaxation that are produced by the orthodontic treatment are important, causing a loss of the tension transmitted on the ligament's surface. Thus, mechanical signals could integrate with other environmental signals and translate into biochemical signals by means of the force-dependent changes in the scaffold geometry. Physical forces of gravity, hemodynamic tensions and movement play a critical role in the tissues, due to the fact that the cells utilize tensegrity architecture for their structural organization.

In conclusion, our results supported the concept that PDL responds to the external mechanical stress produced by orthodontic force. The latter induces a remodeling process. It has been shown that mechanical force applied to human PDL resulted in an increase in the synthesis of laminin, fibronectin and vitronectin. These findings suggest that alterations in the physical environment of cells found in the periodontium can affect biochemical processes, including those that govern the synthesis of structural macromolecules such as ECM protein.

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