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Collagen synthesis from human PDL cells following orthodontic tooth movement*

A. Bumann*, R. S. Carvalho**, C. L. Schwarzer* and E. H. K. Yen***

Departments of Orthodontics, *University of Kiel, Germany, **Harvard School of Dental Medicine, Boston, MA, USA and ***Faculty of Dentistry, University of British Columbia, Vancouver, Canada

SUMMARY The dynamic remodelling processes in the periodontal ligament (PDL) account for the reaction of PDL cells to different orthodontic force simulations. These occur mostly by degradation and synthesis of collagen types I, III, V, VI, XII and XIV. The purpose of this study was to quantify specific collagen types in the PDL from zones of tension and compression of experimental teeth. Such changes could then be correlated with the processes of orthodontic-stimulated tissue breakdown.

Maxillary and mandibular premolars of three females and one male patient were orthodontically moved with a box loop for a total of 14 days, prior to tooth extraction. Teeth from the contralateral side of either the maxilla or the mandible served as the untreated controls. A total of seven experimental and seven control teeth were used in this investigation.

PDL fibroblasts from the cervical third of the roots corresponding to the compression and tension zones of the experimental and control teeth, respectively, were scraped and cultured *in vitro* at 37°C in a humidified incubator with 5 per cent CO_2/95 per cent air. Collagen synthesis of types I, III, V and VI was quantified by using an ELISA.

Application of orthodontic forces in the experimental teeth showed a significant increase ($P < 0.05$) of the synthesis of all collagen types in the compression as opposed to the tension zones. Collagen synthesis on the compression zone of experimental teeth was not significantly different in the mandible when compared with those of the maxilla. In addition, the proportional distribution of different types of collagen was also not significantly different in the PDL fibroblasts from either zone of experimental teeth of either the maxilla or the mandible.

Collagen metabolism in response to orthodontic stimulation appears to be higher in the compression zones and lower in the tension zones. Contrary to what is traditionally assumed in the literature, such findings indicate that in addition to bone resorption, tissue remodelling is very active in zones of compression following the disappearance of the hyalinized areas. These findings constitute a model for future studies on collagen metabolism during orthodontic-stimulated tooth movement.

Introduction

The reaction of the periodontal ligament (PDL) to various orthodontic techniques and the stability of treatment results are basic problems in orthodontics. Orthodontic-induced tooth movement and the maintenance of the ideal tooth position depend on the remodelling of supracrestal gingival and PDL fibres (Edwards, 1988) Regarded as a wound-healing process, the biological cell activity underlying the orthodontic therapy is characterized by a high collagen turnover (Storey, 1973; Ten Cate *et al.*, 1976). This application of forces in the PDL gives rise to the classical histological findings referred to as zones of 'tension' and 'compression' (Storey, 1973). In the compression zone, orthodontic-induced forces generate bioelastic and bioplastic deformations of the alveolar process, resulting in disturbances of circulation, ischaemia and cell death; or areas termed 'hyaline zones'. The altered tissue and the collagen in the hyaline zone are removed by macrophages (Rygh, 1984; Brudvik and Rygh, 1993). The increased cell activity accounts for the

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secretion and formation of new, functionally orientated collagen fibres by migrating PDL fibroblasts (Rygh, 1973). In addition, osteoclasts and PDL fibroblasts form a layer of glycosaminoglycans on the newly resorbed bone surface (Kurihara and Enlow, 1980). On the tension zone, electron microscopy has shown a significant reduction of collagen fibre diameter (Martinez and Johnson, 1987). It is believed that the extension of fibres during the remodelling process results in the elongation of these fibres, allowing for tooth movement (Rygh, 1984).

Although an increased turnover of collagen in the PDL for both the tension and compression zones has been previously shown by several investigators (Crumley, 1964; Koumas and Metthews, 1969; Baumrind and Buck, 1970), the mechanisms of this remodelling process remain unknown. Of special interest is the significance of the various collagen types with respect to orthodontic tooth movement.

PDL as well as extracellular fibres contain different types of collagen as their main component (Bornstein and Sage, 1980). To date 19 collagen types have been identified and classified according to their supramolecular structure (Myers *et al.*, 1994). Among these, collagen types I, III, V, VI, XII and XIV have been found in the PDL (Dublet *et al.*, 1988; Zhang *et al.*, 1993). Collagen type I forms solid fibres anchored to cementum and alveolar bone, providing the PDL with the tensile strength and the capacity to withstand masticatory forces. Collagen type III forms more delicate fibrils accounting for tissue elasticity. Collagen type V is found covering fibrils of types I and III, helping to enhance cell attachment and cell migration (Grotendorst *et al.*, 1981; Martinez- Hernandez *et al.*, 1982). Similarly, the very short microfibrils of collagen type VI link types I and III. Finally, collagen type XII plays a role in the connection of collagen fibres with other components of the extracellular matrix (Oh *et al.*, 1992).

Even though the cellular mechanism of collagen metabolism has been described in detail, the quantitative distribution of distinct collagen types in the orthodontic-treated PDL cells has not been investigated. We believe not only that the orthodontic forces generate a specific pattern of collagen response, but also that the monitoring of individual collagen types will shed some light on the pathological tissue responses induced by orthodontic forces. Therefore, the purpose of this study was to determine the synthesis and relative distribution of collagen types I, III, V and VI from PDL fibroblasts following clinical orthodontic tooth movement.

Subjects and methods

Orthodontic tooth movement

The sample group consisted of three females and a male patient, average age 16 years. Extraction of premolars in both maxilla and mandible was planned for orthodontic purposes. The experimental protocol consisted of moving orthodontically a premolar (experimental) on one side of either the mandible or the maxilla, keeping the contralateral premolar free of stimulation (control) (Table 1). In all patients, anchorage of upper first molars and lower first molars was enhanced by a palatal bar and a Wilson appliance, respectively (Rocky Mountain Orthodontics, Düsseldorf, Germany). Each premolar was moved buccally with a box loop according to Marcotte (1992) for 14 days (Figure 1). These box loops were individually bent from 0.017 × 0.025 inch TMA wire (Ormco Corporation, Lindenberg, Germany). The force

Table 1 Survey of the teeth used in this investigation. In patients 1–3 four premolars were extracted, whereas in patient 4 only two premolars were extracted in the mandible. The numbers in the 'Control' and 'Experimental' columns show the FDI term (Federation Dentaire Internationale) for different teeth.

Patient	Sex	Age	Control	Experimental
Maxilla				
1	female	16	14	24
2	female	12	24	14
3	male	12	24	14
Mandible				
1	female	16	45	35
2	female	12	35	45
3	male	12	35	45
4	female	24	45	35

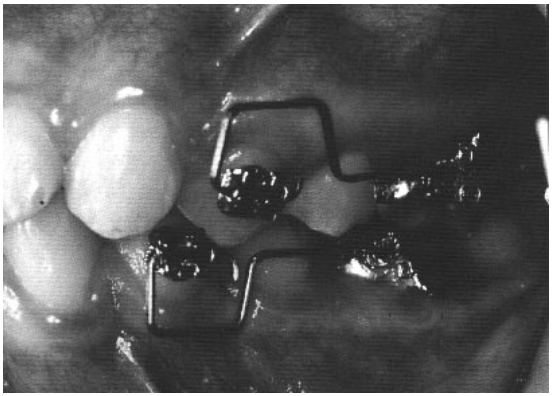

Figure 1 Photograph of a box loop on the right side (mirror picture). The first molars were anchored by a palatal bar and a lingual arch, respectively. Teeth 24 and 34 were moved buccally over a period of 14 days.

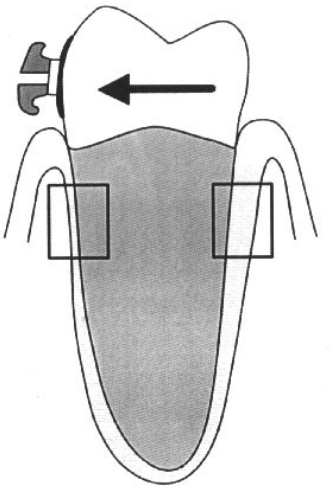

Figure 2 Schematic drawing of the orthodontic tooth movement. The coronal inserted force should move the tooth bodily. The rectangles on the compression (left = buccal) and tension zone (right = lingual) mark the region where the explants after extraction were taken from.

applied was 0.75 N on the upper and 0.90 N on the lower teeth, at the time of loop activation, as checked by a CORREX spring balance (0.1–1 N) (Dentaurum, Pforzheim, Germany). The buccal root surface and the lingual or palatal root surfaces from the experimental teeth were considered as the 'pressure' and 'compression' zones, respectively. Similarly, the buccal root surface and the lingual or palatal root surfaces from the untreated contralateral premolars were considered as the 'control pressure' and 'control compression' zones, respectively.

PDL explants and cell culture

After 14 days of orthodontic tooth movement, both the experimental and control premolars were extracted. These teeth were stored in l-MEM (Biochrom, Berlin, Germany) at 37°C. Approximately 0.5 mm^2 of PDL explants were dissected from the cervical root thirds for both the experimental and control zones (Figure 2). Subsequently, the PDL cells from the different zones of all experimental and control teeth were pooled, respectively, and were cultured without enzymatic treatment according to Ragnarsson *et al.* (1985) at 37°C in a humidified incubator with 5 per cent CO_2/95 per cent air.

All cultures were incubated with α-minimum-essential-medium (α-MEM) (Biochrom) supplemented with 10 per cent heat-inactivated fetal calf serum (FCS) (Nunc, Wiesbaden, Germany), 2 mg/ml sodium hydrogencarbonate (Merck, Darmstadt, Germany), 100 μg/ml ascorbic acid (Merck), 50 mg/ml β-aminoproprionitrile (Sigma, Munich, Germany), 100 U/ml penicillin G (Sigma), 100 mg/ml streptomycin (Sigma) and 40 U/ml of nystatin (Sigma) at pH 7.4. Media were changed every three days and cells were subcultured at a density of 2500 cells/mm^2. Cell passage conditions consisted of incubating cell cultures in a solution of phosphate-buffered saline (PBS), without any Ca^{2+} or Mg^{2+} ions, containing 0.05 per cent trypsin/0.02 per cent EDTA (Nunc). This solution was inactivated with FCS prior to cell centrifugation (1200 r.p.m. for 5 minutes). After resuspension, cell density was 10^5 cells per 3 ml of l-MEM. In each of eight culture dishes, 3 ml of cell suspension was dispensed, for a total of 16 culture dishes per tooth (eight from the compression and eight from the tension zones, respectively).

During the experimental period, cell viability was monitored by trypan blue exclusion. Cells were trypsinized and mixed with the trypan blue solution (50 μl of cell suspension described above with the same amount of trypan blue at 0.5 per cent in physiological sodium chloride).

Subsequently, the cells were counted in triplicate in a Coulter Counter.

At the end of the experimental period, the synthesis for collagen types I, III, V and VI was determined. By establishing the total cell number, the amount of collagen in nanograms and micrograms, respectively, from each experimental zone for each patient used in this study could be calculated.

Collagen synthesis

For determination of collagen synthesis, media was removed from the culture dishes at day 7 of the primary culture. Cells were rinsed three times in PBS and incubated with 3 ml of α-MEM free of FCS. After a 24 hour incubation period, the medium was collected and stored at –20°C prior to carrying out the ELISA technique.

ELISAs were performed in plane microtitre plates (Nunc) as shown in Figure 3. Standard curves were made using purified collagen types I, III, V and VI (all from Southern Biotechnology Associates, USA). The analysis of conditioned medium followed a 1:1 dilution of medium and buffer solution I (15 mM Na_2CO and 35 mM $NaHCO_3$, pH 9.6). One hundred microlitres of this solution was incubated in each well plate for 12 hours at 4°C. Subsequently, the plates were washed three times with buffer solution II (3 mM NaH_2CO_3, 2 mM Na_2HPO_4, 15 mM NaC1, 0.1 per cent Tween 20, pH 7.2) and blocked with buffer solution II containing 1 per cent bovine serum albumin (BSA) (Sigma) for 2 hours at room temperature. Plates were washed again three times in PBS and 100 µl biotin-conjugated primary antibodies against collagen type I, type III, type V or type VI (Southern Biotechnology) all at 1:10,000 dilution in buffer II and incubated for 45 minutes at room temperature on a belly-dancer. The plates were washed again four times in PBS and the wells were incubated with 100 µl of peroxidase-conjugated secondary antibody (biotin–streptavidin antibody (Southern Biotechnology), diluted 1:4000 with buffer solution II, for 45 minutes at room temperature. Substrate (100 ml) ortho-phenyldiamine-dihydrochloride (OPD) (Dakopatts, Hamburg, Germany) dissolved in 12 ml of citrate buffer (38 mM citric acid, 163 mM Na_2HPO_4, pH 5.0) and 5 µl hydrogen peroxide was added to the plates in the dark for 15 minutes. The reaction was terminated with 100 µl of a 1 M sulphuric acid solution.

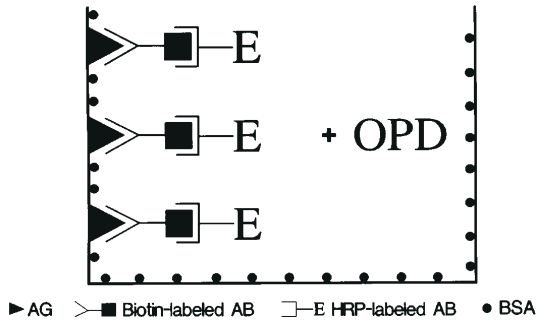

Figure 3 Schematic drawing of the ELISA procedure. AG, antigen; biotin-labelled AB, biotin-conjugated primary antibody; HRP-labelled AB, peroxidase-conjugated secondary antibody; BSA, bovine serum albumin; OPD, ortho-phenylendiamine-dihydrochloride.

The optical density of the enzyme–substrate reaction was measured with a photometer (ELISA-reader) from Behring (Hamburg, Germany) at 490 nm. The data was corrected with the extinction of the wells by using buffer I. All determinations were performed in quadruplicate. Cross-reactivity between antigens and antibodies was determined in preliminary experiments. By using the technique described, it was possible to detect as little as 20 ng of collagen.

Statistical analysis

Mean values of both compression and tension zones from experimental teeth of individual patients were compared with their respective zones from control teeth of the same patient. These values were analysed for statistical differences by unpaired Student *t*-tests. This was repeated for each collagen type. Due to the intrinsic inter-individual variability (age, sex and teeth used) of compression/experimental versus compression/control and tension/experimental versus tension/control, the 'experimental: control' ratio for the compression zone was compared to that of the tension zone for each patient, respectively. *t*-tests were also used to

determine statistical ratio differences for each collagen type studied in each individual patient.

Results

Collagen synthesis on tension and compression zones

In all the control teeth there was no significant difference in the synthesis of collagen types I, III, V and VI between fibroblasts from the buccal (control corresponding to compression zone in experimental teeth) and palatal/lingual (control corresponding to tension zone in experimental teeth) root surfaces (Figure 4). Mean values of each collagen type from ELISA plates corresponding to compression zones of experimental teeth were compared with those of control teeth for every patient. The same was done for the tension zones of experimental versus control teeth. Statistical analysis showed a significant increase ($P < 0.05$) in synthesis of collagen types I, III, V and VI from compression zones in experimental when compared with the same zones in control teeth for all patients (see Figures 4 and 5). Dependent on collagen type, synthesis was increased 1.24–5.0 times (mean 1.73 ± 0.01 and 2.84 ± 1.13) (Table 2). Tension zones in experimental versus control teeth showed no statistical differences in collagen sythesis (see Figures 4 and 5 and Table 2). As indicated above, due to the variability of data among individuals, the mean values for experimental and control teeth for each zone (either compression or tension) were compared as the 'experimental: control' ratio for each zone, respectively. Thus, analysis of these ratios showed a significant increase ($P < 0.05$) in collagen synthesis in the compression zones when compared with the tension zones in each patient (Table 2).

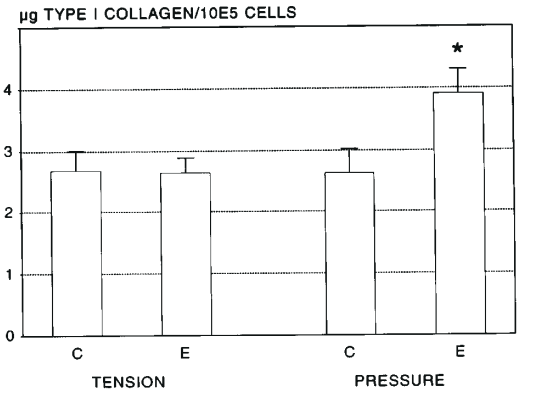

Figure 4 Synthesis of type I collagen on compression and tension zones of lower premolars of a 24-year-old female patient. Collagen synthesis on the pressure side of the experimental tooth (E) was significantly increased compared with the control tooth (C).

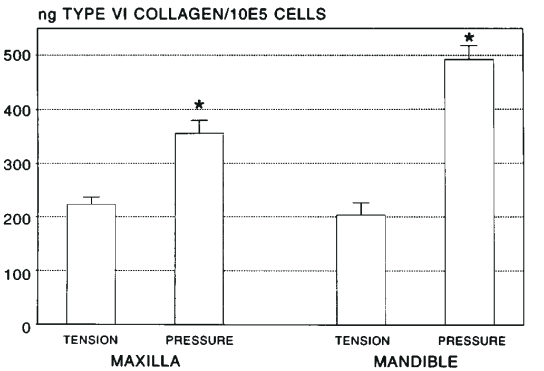

Figure 5 Synthesis of type VI collagen on compression and tension zones of experimental teeth from upper and lower premolars of a 16-year-old female patient. There was a significant increase in collagen synthesis on compression zones in both jaws compared with those of tension zones.

Collagen synthesis from PDL of upper and lower teeth

The mean ratios (experimental:control) for collagen synthesis of each zone (either compression or tension) for the experimental teeth from all patients (average of mean values of all individual patients) for each collagen type was compared in both maxilla and mandible (Table 2). Even though there was a marked difference in values for collagen synthesis in compression versus tension zones, there were no significant differences in collagen synthesis between upper and lower premolars (Table 3).

Differences were expressed as a ratio of collagen synthesis on the compression zones from the upper and lower premolars (upper: lower). In the control teeth, collagen synthesis was higher on the compression zone of upper premolars, whereas in experimental teeth collagen synthesis appeared to be higher at the compression zones of lower premolars when

Table 2 Collagen synthesis of PDL fibroblasts from the compression (P) and tension zones (T). The data show the ratio 'experimental:control' separated for compression and tension zones. Values greater than 1.0 indicate that collagen synthesis in the PDL of experimental teeth was higher than of control teeth. Collagen synthesis in the compression zone was obviously increased in both the maxilla and mandible, whereas in the tension zone only small changes were visible.

Patient	Collagen I		Collagen III		Collagen V		Collagen VI	
	P	T	P	T	P	T	P	T
Maxilla								
1	**1.24***	0.88	**2.64***	1.84	**1.84***	1.12	**1.52***	1.01
2	**2.38***	0.99	**1.79***	0.98	**1.68***	0.98	**1.96***	0.99
3	**1.57***	0.96	**1.29***	1.01	**5.00***	1.30	**1.58***	1.01
Mean	1.73	0.94	1.91	1.28	2.84	1.13	**1.69***	1.00
SD	0.58	0.06	0.68	0.49	1.87	0.16	0.24	0.01
Mandible								
1	**2.45***	1.03	**2.96***	1.37	**1.80***	1.02	**2.38***	0.97
2	**1.67***	0.99	**3.16***	1.06	**2.38***	1.04	**2.62***	1.04
3	**2.34***	1.02	**2.23***	0.99	**2.38***	1.13	**1.62***	0.99
4	**1.47***	0.98	**1.19***	0.93	**3.17***	1.00	**1.86***	0.96
Mean	**1.98***	1.00	**2.39***	**1.09***	**2.43***	**1.05***	2.12*	**0.99***
SD	0.49	0.02	0.89	0.20	0.56	0.06	0.46	0.04

*Values (bold type) represent statistical significance ($P < 0.05$) when the ratio 'experimental:control' of the compression zone for each patient was compared with that of the tension zone of the same patient.

compared with those of upper premolars (Table 3). These results were especially evident for collagen type VI (Figure 5).

Changes in the relative distribution of different collagen types

The percentage amounts of the different collagen types studied here relative to the total collagen in periodontal ligament (= sum of collagen types I, III, V and VI) were calculated from the compression and tension zones of control and experimental teeth. There were no significant differences for all the collagen types in either control or experimental teeth (Figure 6). At the time of sample collection (two weeks after the activation of the orthodontic appliance), the percentage portion of collagen type III was increased, but appeared to be non-significant. Although collagen type I was the highest type synthesized in both com- pression and tension zones from control and experimental teeth, there was a wide range in percentage distribution between the different patients studied (Table 3).

Discussion

Collagen molecules, as the main component of the PDL fibres, play an important role in the remodelling process during orthodontic tooth movement. This study investigated the effects of orthodontic tooth movement on the synthesis of collagen types I, III, V and VI in the human PDL. The following questions were asked: (i) are there any differences in collagen synthesis on the compression and tension zones following orthodontic tooth movement; (ii) are there any differences in orthodontic-stimulated collagen synthesis in the maxillary when compared with the mandibular teeth; and (iii) is there a change in the relative distribution of collagen types I, III, V and VI on both tension and compression zones? The results showed a specific synthesis of all collagen types studied on the compression and tension zones of experimental teeth. However, while collagen synthesis in the tension zone of the treated teeth did not change significantly when compared with the control

Table 3 Comparison of collagen synthesis on compression zones of upper and lower premolars. The values show the ratio 'upper premolar:lower premolar' separated for control teeth (Contr.) and experimental teeth (Exp.). Values greater than 1.0 indicate that collagen synthesis from PDL fibroblasts in the compression zone of upper premolars was higher than in lower premolars. In the experimental group collagen synthesis in the compression zone was higher in lower premoars (value less than 1.0), whereas in the control group collagen synthesis was higher in upper premolars.

	Collagen I		Collagen III		Collagen V		Collagen VI	
Patient	Contr.	Exp.	Contr.	Exp.	Contr.	Exp.	Contr.	Exp.
Maxilla/mandible								
1	1.15	0.58	1.27	1.14	0.92	0.94	1.13	0.72
2	0.63	1.51	0.99	0.56	1.03	0.73	1.10	0.83
3	1.15	0.58	1.27	1.13	0.92	0.94	1.13	0.72

teeth, significantly higher rates of selected types of collagen were found in the corresponding compression zones. This finding was unexpected and according to previous studies in the literature, there seems to be a prevalence of proliferative processes in the tension zones as opposed to degradation (resorption) in the compression zones (Crumley, 1964; Koumas and Metthews, 1969; Baumrind and Buck, 1979). The explanation for such contrasting changes in collagen synthesis from both zones in this study may relate to the activity of proteolytic enzymes, inflammatory wound healing processes, and the type and duration of force application.

The application of orthodontic forces in this study stimulated a particular type of collagen in a particular experimental area (Figures 4 and 5). Collagen type I, III, V and VI investigated appeared to increase significantly in the compression zones of stressed PDL cells. It has been suggested that the mechanism of orthodontically induced PDL remodelling, with an increase in collagen metabolism, resembles wound-healing processes (Clore *et al.*, 1979, Scharffetter *et al.*, 1989). During the early stages of wound healing, collagen expression appears to be time dependent, increasing within hours of the onset of stimulation (Oono *et al.*, 1993). Levels of mRNA expression for collagen types I and III reach their maximum at days 14 and 11, respectively (Oono *et al.*, 1993). However, we have observed that an increase in collagen type VI is not seen before day 11.

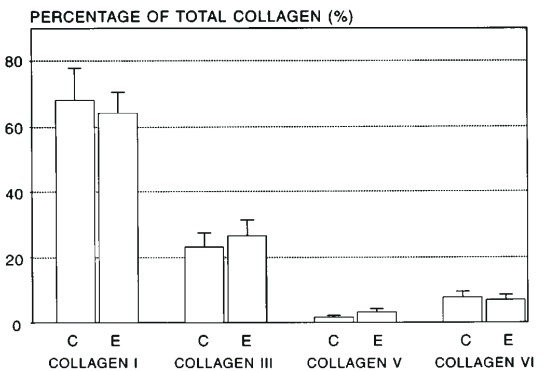

Figure 6 Percentage distribution of different collagen types on the compression side of control (C) and experimental teeth (E). There were no significant differences between control and experimental teeth.

Characterization of collagen types appeared to indicate that the increase in collagen levels reflects an increase in synthesis of collagen types and not an increase in collagenase activity in the compression zone. However, a possible increase in collagenase activity on cells from the tension zone would result in an overall augmentation of collagen synthesis on the compression zone, as seen here. These findings correspond to a correlation between collagenase activity and an increase in proliferative processes, typically seen in tension zones in the PDL following orthodontic stimulation (Green *et al.*, 1990). Collagenase is the only enzyme for resorption of native collagen (Fullmer, 1971). Although the

PDL cells shown here are thought to be capable of secreting collagenase without external stimulation (Nip *et al.*, 1993; Oshima *et al.*, 1993), the significance of collagenase is limited to external catabolism of collagen. It does not participate in phagocytosis or in subsequent intracellular resorption of collagen (Everts *et al.*, 1989). However, the effects of collagenase activity of collagen regulation from orthodontic-stimulated tooth movement were not investigated in this study and clearly require further investigation.

Another intriguing response was the difference in collagen synthesis in both the upper and lower experimental zones of treated teeth. Even though there was no significant difference in collagen synthesis between PDL cells of upper and lower premolars or in the relative distribution of collagen types, the stimulatory response appeared to be higher in the compression zones of lower premolars as compared with upper premolars. Such distribution was best illustrated by changes in collagen type VI (Figure 5). While collagen type VI is found linking fibrils of types I and III, it is not known why any significant changes were not observed in the latter. Even though Yen *et al.* (1989) and others have reported an increase of collagen type III following orthodontic stimulation *in vitro*, changes in collagen type VI found here may represent a regulatory mechanism for both types I and III following orthodontic strain, thus masking any direct effect on types I and III.

Although further study is required on the interaction of the different collagen types as a result of orthodontic stimulation, changes in lower versus upper experimental zones and the variation in the results may also have been caused by changes in the magnitude of forces applied, as previously reported (Lee, 1965; Ricketts *et al.*, 1979). Since the maxillary and mandibular teeth have different root surface areas, the distribution of forces in each zone of individual teeth may differ according to the magnitude of force. Although lower forces were used in this study (0.75 N for upper and 0.90 N for lower premolars), forces of 1 N (Ricketts *et al.*, 1979) and up to 2 N (Lee, 1965) have been reported for experimental tooth movement. In addition, according to finite element models, 1.2–2.6 N of pressure have been suggested to stimulate sufficient pressure during orthodontic tooth movement (Tanne *et al.*, 1987; Cobo *et al.*, 1993).

In conclusion, we have demonstrated that the mechanism of tissue remodeling in the PDL of the compression and tension zones are very complex with regard to collagen synthesis. These findings represent a novel perspective, especially on the reaction of fibroblasts from the compression zones of teeth to mechanical stimulation. We believe that in addition to degradation processes, tissue remodelling is very active in zones of compression following the disappearance of hyalinized areas. The findings described here constitute a model for future studies on collagen metabolism in orthodontic-stimulated tooth movement. Ultimately, collagen changes induced both physiologically and orthodontically could be correlated in order to understand better the changes involved in tissue breakdown.

Address for correspondence

Prof. Dr Axel Bumann
Klinik für Kieferorthopädie
Christian-Albrechts-Universität
Arnold-Heller-Straße 16
D-24105 Kiel
Germany

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