J Periodont Res 2004; 39; 27–32
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JOURNAL OF PERIODONTAL RESEARCH

The effect of centrifugal force on mRNA levels of collagenase, collagen type-I, tissue inhibitors of metalloproteinases and β-actin in cultured human periodontal ligament fibroblasts

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Redlich M, Roos H, Reichenberg E, Zaks B, Grosskop A, Bar Kana I, Pitaru S, Palmon A. The effect of centrifugal force on mRNA levels of collagenase, collagen type-I, tissue inhibitors of metalloproteinases and β-actin in cultured human periodontal ligament fibroblasts. J Periodont Res 2004; 39; 27–32. © Blackwell Munksgaard, 2004

Background: The aim of orthodontic treatment is to relocate teeth abnormally positioned in the jaws. This is achieved by application of continuous force on the tooth, which is immediately being sensed by the periodontal ligament (PDL), bone and the gingiva. Since the bony response is mediated by the PDL, tooth movement is primarily a PDL phenomenon.

Objectives: Thus, the purpose of the present study was to evaluate the direct effect of force (excluding the *in vivo* tissue response) on the molecular level of matrix metalloproteinase-1 (MMP-1) and collagen type-I (Col-I) in human PDL fibroblasts.

Methods: PDL cell culture flasks were centrifuged for 10, 20, 30, 60, 90 and 120 min by horizontal microplate rotor. The effect of force on mRNA levels of β-actin, MMP-1, Col-I, tissue inhibitors-1 and -2 (TIMPs) genes was analyzed by RT-PCR.

Results: The results showed that force had no effect on the mRNA levels of β-actin during the first 90 min of application of force, indicating for the first time the use of β-actin gene as an internal invariant control. It increased the mRNA levels of MMP-1 while almost no effect on Col-I and TIMPs was observed.

Conclusions: The results indicate that PDL remodeling following application of orthodontic force could be partly attributed to the direct effect of the force on MMP-1 gene expression in fibroblasts.

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Key words: gene expression; matrix metalloproteinase-1; orthodontic force; periodontal ligament

Accepted for publication June 9, 2003

The periodontal ligament (PDL) provides anchorage and support to the functioning tooth. Its extracellular matrix (ECM) consists predominantly of collagen type-I (Col-I), whereas fibroblasts are the most abundant cells in the PDL (1). The turnover of the collagen fibers in the PDL is very rapid (2). This steady-state homeostasis implicates towards intensive and subtle transcriptional and translational regulation of the collagens and the matrix metalloproteinases (MMPs) genes. MMPs, a family of zinc-dependent enzymes, have the capacity to degrade almost all components of the ECM (3). Within this family of enzymes, MMP-1 initiates cleavage of fibrillar collagens at a single site, a prerequisite process for further degradation of the cleaved fragments by other MMPs.

The aim of orthodontic treatment is to relocate teeth abnormally positioned in the jaws. This is achieved by application of continuous mechanical force on the tooth, which immediately affects the PDL, the bone and the gingiva. The immediate effect of an *in vivo* orthodontic force is dual: (i) injury to the PDL and a subsequent wound-healing process and (ii) exertion of mechanical force on the cell. Thus, the cells in the PDL simultaneously respond to two different events: ECM damage and mechanical force. The latter is assumed to be sensed by cell membrane components and then transduced intracellulary into biological signals.

The dental attachment apparatus, which responds to orthodontic forces, is exposed to pressure-type forces (in the direction of tooth movement) and tension-type forces (the opposite side of the pressure) leading to remodeling of the PDL, the bone and the gingiva which enables the transposition of the tooth (4).

Recently, we showed that following application of force the *in vivo* molecular equilibrium between collagen synthesis and degradation in the gingiva is disturbed (5). Both pressure and tension vectors of the force cause a significant increase in the mRNA levels of MMP-1, as well as in its interstitial activity.

Separating the dual effect of the orthodontic force (tissue injury and cell–force interaction) is impossible in an *in vivo* system and therefore only an *in vitro* model is capable of simulating genuine cells–force interactions. Also, the *in vitro* model should differentiate between pressure and tension types of force. In an earlier study we established an *in vitro* pressure model by centrifugation of cell culture (6). The advantage of the centrifugal pressure model, as well as the other *in vitro* tension systems (7, 8), is that it evaluates the direct effect of pressure or tension on the cells excluding the *in vivo* tissue response to the force. Consequently, direct correlation is established between pressure/tension force and phenotypic cellular response. Since the bony response is mediated by the PDL, tooth movement is primarily a PDL phenomenon. Thus, the purpose of the present study was to evaluate the molecular MMP-1 and Col-I interactions of human PDL fibroblasts subjected to an *in vitro* continuous pressure type of mechanical force.

Materials and methods

Cell culture

Human PDL fibroblasts were isolated from healthy PDL of premolar teeth of individuals undergoing tooth extraction for orthodontic treatment in accordance with the method of Somerman *et al.* (9), with minor modifications as we previously published (10, 11). All the patients gave informed consent before providing the samples. Healthy periodontal tissue was removed from the center of the root surface with a surgical scalpel. The tissue was minced, and then transferred to plastic Leighton tubes (Costor, Cambridge, MA, USA). The explants were cultured in α-MEM supplemented with 10% fetal calf serum, 50 U/ml penicillin G and 50 μg/ml streptomycin (henceforth denoted standard medium) with changing medium every 2 or 3 days. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2. When the cells growing out from the explants had reached confluence, they were separated by treatment with 0.05% trypsin–0.53 mM ethylenediaminetetraacetic acid (EDTA), collected by centrifugation, and cultured on culture plastic dishes containing the standard medium until confluency. The cells were then trypsinized at 1 : 3 split ratio. Experiments were carried out with cells from the second (p2) or third (p3) passages. In this study, for each experiment, we used explants from at least three different volunteers.

Application of centrifugal force *in vitro*

Cell culture flasks ($n > 3$) were centrifuged at 167 *g* for 10, 20, 30, 60, 90 and 120 min by horizontal microplate rotor as previously described (6). This model is based on application of a constant pressure centrifugal force at a magnitude of 33.5 g/cm^2, which resembles clinical orthodontic force (4).

The calculation of force is based on the equation:

$$P=(m\times r\times \text{r.p.m.}^2\times\pi^2)/(A\times 9.8\times 900),$$

where P = kg pressure per cm^2 of cells, m = mass of medium (0.005 kg), r = radius (0.15 m), r.p.m. = revolu-evolution/min (1000), A = area of contact between medium and cells (25 cm^2).

Trypan blue cell vitality exclusion test

Trypan blue cell vitality exclusion test was performed as we previously described (6).

Cell collection and RNA extraction

Monolayer cell cultures were suspended in TRI-REAGENT (Molecular Research Center, Cincinnati, USA). Total RNA was extracted as previously described (10, 11).

Determination of mRNA levels of Col-1, MMP-1, TIMP-1 and TIMP-2 by RT-PCR

To measure mRNA levels of β-actin, Col-I, MMP-1, tissue inhibitors-1 and -2 (TIMP-1 and TIMP-2), semi-quantitative RT-PCR analysis was performed for each gene transcript as previously described (5, 10, 11). Primer sequences and the optimal conditions for each set of primers derived

from calibration curves are presented in Table 1 and Figs 1(a and b). Actin was used as an internal invariant control subsequent to providing data about its non-dependent expression following application of force (see Results). When calculating the effect of force we normalized the results to actin mRNA levels and therefore the effect of force is relative to housekeeping gene actin.

Statistical analysis

For statistical analysis, student's unpaired *t*-test was used for individual matched-group comparisons. Data are expressed as means ± SE. Values of $p < 0.05$ were considered to be statistical significant.

Results

The effect of centrifugal force on mRNA levels of β-actin

Because no data is available on the effect of pressure on mRNA levels of β-actin, in initial experiments, the mRNA levels of β-actin expressed by PDL cells in culture were determined following application of different durations of centrifugal force and compared to control (non-centrifuged) cultures. In the first 90 min of application of centrifugal force almost no change in the β-actin mRNA levels was found (Fig. 2), thereafter at 120 min a decrease of 20% below control levels was detected (Fig. 2). By that time (120 min), 80% cell vitality was shown by the vitality exclusion test. The 18S ribosomal RNA stained by ethidium bromide on agarose gel paralleled cell vitality results. The average ($n = 57$) experimental to control β-actin mRNA levels in the first 90 min was calculated to be 0.99 ± 0.13.

The effect of centrifugal force on mRNA levels of MMP-1, Col-I, TIMP-1 and TIMP-2

To monitor the kinetic effect of force on PDL cells, cell culture flasks were subjected to centrifugal force for 10, 20, 30, 60 and 90 min and mRNA levels were determined. After 30 min the mRNA levels of MMP-1 (compared to control and normalized to β-actin) increased significantly ($p < 0.05$) to a peak level of 2.6-fold. Thereafter, at 60 min, the mRNA levels decreased but still remained higher than the control levels and at 90 min mRNA levels decreased almost to control levels (Fig. 3).

The mRNA levels of Col-I, TIMP-1 and TIMP-2, showed the same pattern as MMP-1, but in a lesser degree: in the first 60 min of application of force there was an increase to peak levels, thereafter at 90 min there was a decrease to almost control levels. The peak levels for Col-I and TIMP-1 were found to be about 1.25, and 1.6 for TIMP-2.

Discussion

Actins constitute a family of highly conserved proteins found in all eukaryotic cells. Whitin this family, α-actins are the muscle actins involved in muscle contraction and β-actins are known as the cytoplasmic type found in many non-muscle cells (12). The β-actin filaments are involved in cell motility, cell morphology and various cytoskeletal functions such as transduction of extracellular stimuli into cellular responses (13). Although β-actins are commonly used as invariant internal control in many mRNA expression studies, we could not find a conclusive report showing that mechanical stress does not affect mRNA levels of fibroblasts cytoplasmatic β-actin.

The results of the present study show no changes in mRNA levels of β-actin during the first 90 min of application of pressure on PDL fibroblasts. This finding was also accompanied with similar amounts of intact 18S ribosomal RNA and cell vitality levels, emphasizing that β-actin mRNA levels followed cell vitality and were not altered by the force. It verifies, for the first time, the use of β-actin gene as invariant control in fibroblasts reponse to mechanical loading.

A recent study (14), in which tensile mechanical loading was applied on tendon cells, showed no changes in the amount of cytoskeletal actin stress fibers at the protein level. However, a significant spatial organization of these fibers was inflicted by the force.

ECM remodeling is a highly regulated complex process, which control the homeostasis and repair of connective tissues such as the PDL following application of mechanical orthodontic force. The initial effect of the

Table 1. Sequences of primer sets

Primers	Sequences	Annealing (°C)	Mg (mM)	Length (bp)	Reference
MMP-1	5′-TGGGAGCAAACACATCTGA-3′ (sense) 5′-ATCACTTCTCCCCGAATCGT-3′ (anti-sense)	48	2.5	560	Aust *et al.* (24)
Collagen type-1α1	5′-CTGGCAAAGAAGGCGGCAAA-3′ (sense) 5′-CTCACCACGATCACCACTCT-3′ (anti-sense)	62	1.0	502	Spotila *et al.* (25)
TIMP-1	5′-ATCCTGTTGTTGCTGTGGCTGATAG-3′ (sense) 5′-TGCTGGGTGGTAACTCTTTATTTCA-3′ (anti-sense)	60	1.0	689	Alvares *et al.* (18)
TIMP-2	5′-GGAAGTGGACTCTGGAAACGACATT-3′ (sense) 5′-CTCGATGTCGAGAAACTCCTGCTTG-3′ (anti-sense)	62	2.0	495	Nomura *et al.* (26)
Actin	5′-GAGACCTTCAACACCCCAGCC-3′ (sense) 5′-GGCCATCTCTTGCTCGAAGTC-3′ (anti-sense)	62	1.0	311	Palmon *et al.* (27)

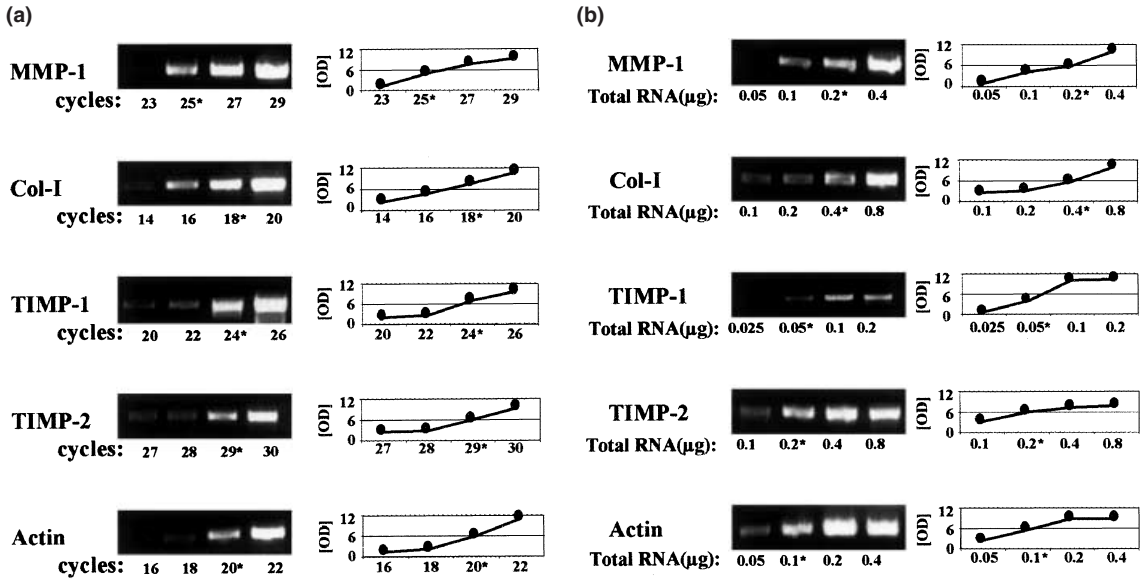

Fig. 1. Calibration curves for MMP-1, Col-I, TIMP-1, TIMP-2, and β-actin. Total RNA (1 μg) was reverse transcribed using oligo $(dt)_{15}$ primer. cDNA was amplified by PCR using the relevant primers. (b) input total RNA amplified with the PCR cycles determined by (a). Optimal conditions used for each amplification primer are marked with *.

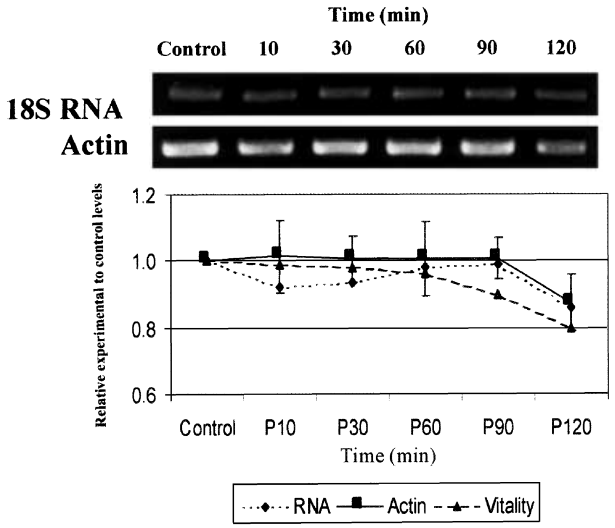

Fig. 2. 18 S RNA, actin PCR-amplified mRNA levels and cell vitality of control and force-affected cells. Upper panel show ethidium bromide stained agarose gel of 18S RNA and actin PCR-amplified bands. Lower graphs show relative optical density (OD) to control of 18S RNA and actin PCR-amplified bands at each time point and cell vitality data.

orthodontic pressure on PDL is tissue damage, which will later affect the adjacent alveolar bone (4). Temporal concerted cells–ECM interactions, as well as the response of cells to extracellular signals are essential in tissue remodeling. Consequently, regulated response of ECM proteins (collagens) on the one hand and MMPs and their inhibitors, TIMPs, on the other hand is required for this process to occur properly (15). The present study focused on the molecular response of human PDL fibroblasts to centrifugal pressure, which simulates the commonly used orthodontic force. The conspicuous finding of this study is the time-dependent up-regulation of mRNA levels of MMP1 in the first 30–60 min of application of force. This effect was time limited and was followed by a decrease of MMP-1 mRNA almost to control levels after 90 min of force application. This time-limited phenomenon, which was also found *in vivo* (5), highlights the importance of not only MMP-1 up regulation in response to force but also the control-limiting mechanism of MMP-1 gene necessary for maintaining proper tissue integrity. Interestingly, the time course of MMP-1 induction and normalization in the *in vitro* model was by far more rapid than in the *in vivo* model, which corresponds to the high turnover rate of PDL cell culture model used in this study (2). In the present study we focused on the immediate response, bearing in mind that it is a part of a broader phenomenon. We are currently investigating longer force induction periods.

To prove the principle that centrifugal pressure in an *in vitro* model induces changes in the pathway/s of extracellular trigger to mRNA transcription we used the semi-quantitative RT-PCR assay that we previously established (6). The results found are within the scope of this method. Other methods, such as real-time PCR or zymography, may be needed in the future to further quantitatively characterize this phenomenon. However, such an accurate determination is not needed for utilizing this model to elu-

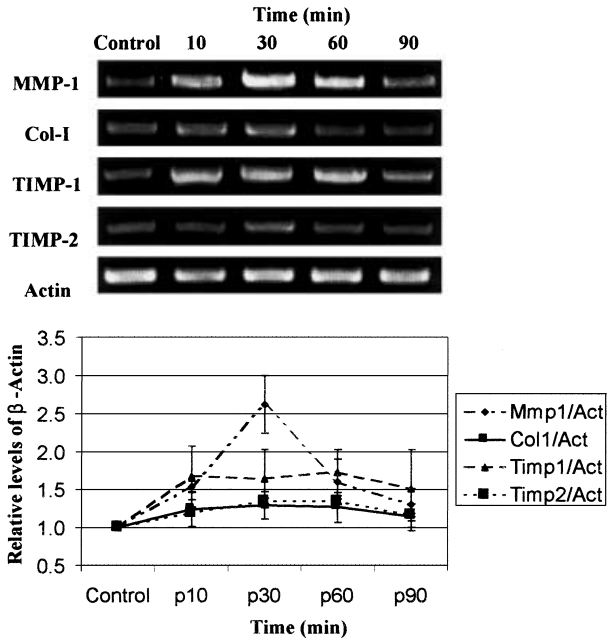

Fig. 3. Semi-quantitative RT-PCR assay of MMP-1, Col-I, TIMP-1 and TIMP-2 following application of force. Upper panels show representative set of PCR bands after gel electrophoresis. Lower graphs show the ratio between OD of force to OD of control at each time point after normalization to β-actin (for details see text). Each time point represents the mean ± SE.

cidate the cellular underlying mechanisms induced by centrifugal pressure.

The increase in MMP-1 mRNA, concomitant with no changes in mRNA levels of TIMPs indicate that elevated levels of MMP-1 protein might be synthesized leading to increased PDL remodeling. A similar process occurred in the *in vivo* model (5), in which orthodontic pressure caused significant induction of gingival mRNA levels of MMP-1 followed by increased protein activity.

Since an inevitable consequence of orthodontic intervention is that of tissue injury and subsequent inflammatory response, one may assume that PDL degradation is the result of the inflammatory response, during which collagenase is highly expressed by polymorphonuclear cells, macrophages and by fibroblasts that invade the injured area (16). The major finding of the present study that force triggers MMP-1 gene expression indicates that PDL remodeling, following application of force, is the cumulative result of both the force affecting the cells and of the inflammatory process.

The responsiveness of MMP-1 gene to force, as shown in the present work, supports previously described changes in the expression of MMP-1 genes under the influences of various cellular and extracellular factors such as alteration in actin cytoskeleton (17), growth factors (18), chemical agents (19) and physical stress (20). It also corresponds to a recent *in vitro* tension model in which both PDL and gingival human fibroblasts over expressed mRNA encoding for MMP-1 (21).

The precise regulation of MMP gene expression in relation to collagen gene expression is critical for tissue repair and homeostasis. Non-coordinated changes in the expression of each of the two genes may lead to pathologic events such as keloids formation due to over expression of Col-I (22) or to certain ulcerative skin lesions due to over expression of MMP-1 (23). Therefore, at the molecular level, the amount and timing of expression of each of these genes is essential for proper tissue repair. However, the relatively short duration of centrifugation in this study (due to cell death), as compared to the *in vivo* model (5), does not provide sufficient data for evaluating the force interdependent changes between MMP-1 and Col-I mRNAs.

This issue, as well as tracking possible pathways involved in the mechano-signal transduction processes, is the objective of our current investigations.

Acknowledgements

This research was supported in part by the Israel Science Fund (grant number: 611/02–1) and in part by Israel Ministry of Health (grant number: 5006).

References

1. Berkovitz BKB. The structure of the periodontal ligament: an update. *Eur J Orthod* 1990;**12:**51–76.
2. Sodek J, Limeback HF. Comparison of the rates of synthesis, conversion and maturation of type I and type III collagens in rat periodontal tissues. *J Biol Chem* 1979;**254:**10496–10502.
3. Shapiro SD. Matrix metalloproteinase degradation of extracellular matrix: Biological consequences. *Curr Opin Cell Biol* 1998;**10:**602–608.
4. Davidovitch Z. Tooth movement. *Crit Rev Oral Biol Med* 1991;**2:**411–450.
5. Redlich M, Reichenberg E, Harari D, Zaks B, Shoshan S, Palmon A. The effect of mechanical force on mRNA levels of collagenase, collagen type I and tissue inhibitors of metalloproteinases in gingivae of dogs. *J Dent Res* 2001;**80:**2080–2084.
6. Redlich M, Palmon A, Zaks B, Geremi E, Rayzman S, Shoshan S. The effect of centrifugal force on the mRNA levels of collagen type I and collagenase in cultured canine gingival fibroblasts. *Arch Oral Biol* 1998;**43:**313–316.
7. Lambert CA, Soudant EP, Nusgens BV, Lapiere CM. Pretranslational regulation of extracellular matrix macromolecules and collagenase expression in fibroblasts by mechanical force. *Lab Invest* 1992;**66:**444–451.
8. Kletsas D, Basdra EK, Papavassiliou AG. Effect of protein kinase inhibitors on the stretch-elicited c-Fos and c-Jun up-regulation in human PDL osteoblast-like cells. *J Cell Physiol* 2002;**190:**313–321.
9. Somerman MJ, Archer SY, Imm GR, Foster RA. A comparative study of human periodontal ligament cells and gingival fibroblasts in vitro. *J Dent Res* 1988;**67:**66–70.

10. Palmon A, Roos H, Edel J, *et al*. Inverse biphasic and dose depended effect of bFGF on the gene expression of collagen type I and MMP-1 by periodontal ligament cells in culture. *J Periodontol* 2000;**71:**974–980.
11. Palmon A, Roos H, Grosskop A, Bar-Kana I, Pitaru S, Redlich M. Basic fibroblasts growth factor suppresses tropoelastin gene expression in cultured human periodontal fibroblasts. *J Periodont Res* 2001;**36:**65–70.
12. Czosnek H, Nudel U, Mayer Y, *et al*. The genes coding for cardiac muscle actin, the skeletal muscle actin and the cytoplasmatic β-actin are located on three different mouse chromosomes. *EMBO J* 1983;**2:**1977–1979.
13. Defilippi P, Olivo C, Venturino M, Dolce L, Silengo L, Tarone G. Actin cytoskeleton organisation in response to integrin-mediated adhesion. *Microsc Res Tech* 1999; **47:**67–78.
14. Ralphs JR, Waggett AD, Benjamin M. Actin stress fibers and cell–cell adhesion molecules in tendons. organisation in vivo and response to mechanical loading of tendon cells in vitro. *Matrix Biol* 2002;**21:**67–74.
15. Reynolds JJ. Collagenases and tissue inhibitors of metalloproteinases: a functional balance in tissue degradation. *Oral Dis* 1996;**2:**70–76.
16. Shoshan S. Wound healing. In: Hall, DA, Jackson, DS, eds. *International review of connective tissue research*. New York: Academic Press, 1981: 1–26.
17. Langholtz O, Rockel D, Mauch C, *et al*. Collagen and collagenase gene expression in three-dimensional collagen lattices are differentially regulated by alpha 1 beta 1 and alpha 2 beta 1 integrins. *J Cell Biol* 1995;**131:**1903–1915.
18. Alvares O, Klebe R, Grant G, Cochran DL. Growth factor effects on the expression of collagenase and TIMP-1 in periodontal ligament cells. *J Periodontol* 1995; **66:**552–558.
19. McCarty GM, Mitchell PG, Cheung HS. The mitogenic response to stimulation with basic calcium phosphate crystals is accompanied by induction and secretion of collagenase in human fibroblasts. *Arthritis Rheum* 1991;**34:**1021–1030.
20. Prajapat RT, Chavally-Mis B, Herbage D, Eastwood M, Brown RA. Mechanical loading regulates protease production by fibroblasts in three-dimensional collagen substrates. *Wound Repair Regen* 2000;**8:** 22–37.
21. Balcato-Bellemin AL, Elkaim R, Abehsera A, Fausser JL, Haikel Y, Tenenbaum H. Expression of mRNAs encoding for α and β integrin subunits, MMPs, and TIMPs in stretched human periodontal ligament and gingival fibroblasts. *J Dent Res* 2000;**79:**1712–1716.
22. Uitto J, Perejda AJ, Abergel RP, Chu ML, Ramirez F. Altered steady-state ratio of type I/III procollagen mRNAs correlates with selectively increased type I procollagen biosynthesis in cultured keloid fibroblasts. *Proc Natl Acad Sci USA* 1985; **82:**5935–5939.
23. Saarialho-Kere UK, Chang ES, Welgus HG, Parks WC. Expression of interstitial collagenase, 92-kDa gelatinase, and tissue inhibitor of metalloproteinases-1 in granuloma annulare and necrobiosis lipoidica diabeticorum. *Invest Dermatol* 1993; **100:**335–342.
24. Aust G, Hofmann A, Laue S, Rost A, Kohler T, Scherbaum WA. Human thyroid carcinoma cell lines and normal thyrocytes: expression and regulation of matrix metalloproteinase-1 and tissue matrix metalloproteinase inhibitor-1 messenger-RNA and protein. *Thyroid* 1997;**7:**713–724.
25. Spotila LD, Colige A, Sereda L *et al*. Mutation analysis of coding sequences for type I procollagen in individuals with low bone density. *J Bone Miner Res* 1994; **9:**923–932.
26. Nomura T, Takahashi T, Hara K. Expression of TIMP-1, TIMP-2 and collagenase mRNA in periodontitis-affected human gingival tissue. *J Periodont Res* 1993;**28:**354–362.
27. Palmon A, Ben-Aroya N, Tel-Or S, Burstein Y, Fridkin M, Koch I. The gene for the neuropeptide gonadotropine-releasing hormone is expressed in the mammary gland of lactating rats. *Proc Natl Acad Sci USA* 1994;**91:**4994–4996.