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Cultured human periodontal ligament cells constitutively express multiple osteotropic cytokines and growth factors, several of which are responsive to mechanical deformation

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Background and Objective: A role for cytokines and growth factors in mediating the cellular and molecular events involved in orthodontic tooth movement is well established. The focus to date, however, has been largely on individual mediators, rather than to study cytokines in terms of complex interacting networks. Our objective was to expand our knowledge of the cytokines and growth factors expressed by human periodontal ligament (PDL) cells and to identify new genes that are responsive to mechanical deformation.

Material and Methods: Human PDL cells were strained with a cyclic deformation of 12% for 6–24 h, and the differential expression of 79 cytokine and growth factor genes was quantified using real-time RT-PCR arrays. For statistical comparison, *t*-tests were used with mean critical threshold (CT) values derived from triplicate samples.

Results: Forty-one genes were detected at CT values < 35 and, of these, 15 showed a significant change in relative expression. These included seven interleukins (IL): *IL1A*, *IL1F7*, *IL6* and *IL7* (down), *IL8*, *IL11* and *IL12A* (up). Eight genes representing other cytokine and growth factor families showed comparable mechanical sensitivity, including *VEGFD* and *OPG* (down) and *PDGFA*, *INHBA*, *GDF8* and two transforming growth factor β genes, *TGFB1* and *TGFB3* (up). The genes *CSF2*/*GMCSF and IL11* were found to be consistently stimulated across all three time points. Genes that were not expressed included: (1) the immunoregulatory lymphokines (*IL2–IL5*), *IL17* and *IL17B*; (2) *IL10* and other members of the IL-10 family of anti-inflammatory cytokines (*IL19*, *IL20*, *IL22* and *IL24*); and (3) *TNF* and *RANKL*.

Conclusion: Human PDL cells constitutively express numerous osteotropic cytokines and growth factors, many of which are mechanoresponsive.

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An important role for cytokines and growth factors in mediating many of the cellular and molecular events in connective tissue turnover and periodontal ligament (PDL) remodelling is now well established. The first direct evidence for cytokine involvement in orthodontic tooth movement was provided by Davidovitch *et al.* (1), when interleukin-1β (IL-1β) was immunolocalized in the PDL of cats. Human PDL cells have since been widely used to study the effects of mechanical strain on cytokine expression *in vitro* (2–5), and clinical investigations have shown that IL-1β, tumour necrosis factor-α (TNF-α), IL-6 and epidermal growth factor (EGF) are elevated in the gingival crevicular fluid of patients during orthodontic treatment (6–8). Rat models of tooth movement have provided additional *in vivo* evidence that IL-1β, IL-6 and TNF-α are upregulated in PDL cells and osteoblasts (9,10), as are collagenase-2 (MMP-8) and collagenase-3 (MMP-13; 11).

Receptor activator of nuclear factor-κB ligand (RANKL), its membrane receptor RANK and osteoprotegerin (OPG), a decoy receptor for RANKL, are key molecular determinants of osteoclast formation and function (12–14), and accumulating evidence suggests that RANKL and OPG play important roles in PDL turnover and bone resorption during orthodontic tooth movement. Kanzaki *et al.* (15) reported that culture media from compressed human PDL cells stimulated osteoclast formation *in vitro* and showed that while RANKL expression was upregulated, the expression of OPG remained unchanged. Recent reports, however, suggest that both OPG and RANKL mRNA expression are upregulated in human PDL cells by cyclic tension (16,17) and by hydrostatic pressure (18). Both RANKL and OPG have been shown to be expressed in rat periodontal tissues, and positive signals for RANKL and RANK detected in osteoclasts at sites of bone resorption during tooth movement (19). Most recently, microarray analyses have been used to study gene expression in PDL cells in conditions of both tension and compression (20–22). The aim of the present investigation was to expand our knowledge of the cytokines and growth factors expressed by human PDL cells *in vitro* using targeted real-time reverse transcription polymerase chain reaction (RT-PCR) arrays, and to identify additional genes that might be responsive to mechanical deformation.

Material and methods

Preparation of human PDL cells

Human PDL cells were prepared from premolar teeth extracted for orthodontic reasons as described previously (23), approval having been granted by the University of Otago Ethics Committee (Reference: 05/069) to harvest the teeth with the consent of the donor and/or parent. Teeth were washed with phosphate-buffered saline (PBS) and the PDL attached to the middle third of the root removed with a scalpel. Tissue explants were plated onto 1.9 cm^2, 24-well Nunclon multidishes in Dulbecco's modification of Eagle's medium (DMEM; Gibco, Invitrogen, Penrose, New Zealand) supplemented with 10% fetal calf serum (Gibco) and antibiotic–antimycotic reagent (10,000 units penicillin, 10,000 μg streptomycin and 25 μg/mL amphotericin B; Invitrogen), 100 mMol L-glutamine (Invitrogen) and Gentamicin reagent solution (10 mg/mL, Gibco) and cultured at 37°C in a humidified atmosphere of 5% CO_2–95% air. Confluent cultures were lifted with trypsin-EDTA (Gibco) and passaged through a progression (25, 75 and 175 cm^2) of tissue culture flasks (Cellstar; Greiner Bio-One AG, Monroe, NC, USA). Stocks of cells were frozen in Cell Culture Freezing Medium (Gibco) at −80°C and then transferred to liquid nitrogen for long-term storage. Several PDL cell lines have been established, and fourth passage cells from a single patient were used in this study.

Application of tensile strain to PDL cells

Human PDL cells (3×10^5 per well) were subcultured into 6-well, 35 mm flexible-bottomed Uniflex culture plates with a centrally located, rectangular type I collagen-coated culture strip (15.25 mm × 24.18 mm), designed to provide a uniform uniaxial strain. The cells were strained with a cyclic in-plane substrate deformation of 12% for 6 s every 90 s with a Flexercell FX-4000 Strain Unit (Flexcell Corporation, Hillsborough, NC, USA). Uniaxial strain was chosen to more closely represent the deformation to which PDL cells are exposed during orthodontic tooth movement and the strain value of 12% selected on the basis of numerical data derived from a finite element model; this suggested that maximal PDL strains for horizontal displacements of a human maxillary central incisor under physiological loading conditions lies in the vicinity of 8–25%, depending upon the apico-crestal position. A value of 12% correlates well with strain conditions predicted at the mid-root (24). The strain protocol was based on a pilot study that compared cyclic deformation (as used in this investigation) with another designed to more closely emulate loading conditions *in vivo*; this consisted of a continuous strain of 6% to reproduce the effect of an orthodontic appliance on the PDL, and was interrupted by an additional deformation of 6% every 20 s to mimic the effects of occlusal loading. We found that the differential expression of three test genes (*OPG*, *CSF1* and *IL1B*) was independent of the strain regime, suggesting little difference between the two experimental protocols at the biological level (MN Pinkerton, DC Westcott, BJ Gaffey, KT Beggs, TJ Milne, MC Meikle, unpublished findings). Four plates (two experimental and two control) were allocated to each of the three time intervals (6, 12 and 24 h). The Flexercell strain unit was paused temporarily to allow plate removal at the end of each experimental time period. Plates were then examined under the light microscope to determine changes in the orientation of cultured cells and to inspect plates for evidence of culture detachment or delamination.

Isolation of RNA

Culture media were removed from the wells prior to total RNA isolation by a

modification of the method of Chomczynski & Sacchi (25). Briefly, 0.5 mL of Trizol reagent (Invitrogen) was added to each well of the Uniflex plate. After a 5 min incubation period, the cell lysate was added to a tube containing 0.1 mL of chloroform and shaken vigorously by hand for 15 s. A further incubation of 2–3 min at room temperature was required prior to centrifugation of the samples at 12,000 × *g* for 15 min at 4°C. Following centrifugation, 300 μL of the clear aqueous phase was added to an equal volume of 70% ethanol and vortexed to disperse the precipitate. Sample purity was achieved using the Purelink Micro-to-Midi Total RNA Purification System (Invitrogen) in accordance with the manufacturer's instructions. Total RNA samples were eluted from the columns in 50 μL of RNase-free water and stored at −80°C. The concentration and purity (ratio of absorbance at 260 and 280 nm, A_{260}/A_{280}) of the samples was determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Rockland, DE, USA).

Real-time RT-PCR microarray analysis

Total RNA samples were analysed by Superarray Bioscience Corporation (Fredrick, MD, USA). Contaminating genomic DNA was removed from total RNA samples by Dnase I digestion prior to first strand synthesis. First strand synthesis was performed using the RT^2 PCR array First Strand Kit. Samples were then screened for the expression of 79 genes encoding common cytokines using the RT^2 Profiler PCR Array System. Experimental and control samples at each of the three time points were analysed in triplicate to allow for biological variation between samples and provide a statistically sound data set.

Statistical methods

Expression profiles of the target genes were measured relative to the mean critical threshold (CT) values of five different calibrator genes (*GAPDH*, *B2M*, *ACTB*, *HPRT1* and *RPLI3A*) using the ΔΔCT method described by Livak & Schmittgen (26). For statistical comparison of the control and experimental groups, *t*-tests were performed using mean CT values derived from the triplicate samples (27).

Results

Assessment of the integrity and orientation of the mechanically deformed cells by light microscopy showed that all cultures remained intact with no evidence of detachment or delamination at any point in the time scale. The responsiveness of the cells to the magnitude and frequency of the applied force was evident by 6 h; cells in the experimental group had started to re-orientate away from the direction of the applied force as previously reported for primary human skin fibroblasts (28).

The 79 target genes screened using RT-PCR arrays are shown in Table 1. More than half of those included ($n = 41$) were detected at a CT value of less than 35, reflecting a significant level of basal expression. A further 38 genes were detected at CT values greater than 35, placing them at the limits of sensitivity of the system, and were therefore considered not to have been expressed. These included: (1) interleukin-1 family members (*IL1F5–IL1F10*) with the exception of *IL1F7*, which was not only expressed, but also significantly downregulated by mechanical deformation; (2) several powerful immunoregulatory lymphokines (*IL2–IL5*), *IL17* and *IL17B*; and (3) *IL10* and other members of the IL-10 family of cytokines with anti-inflammatory properties (*IL19*, *IL20*, *IL22* and *IL24*). Another two genes found not to have been expressed were *TNF* and *RANKL/TNFSF11*.

In response to the intermittent tensile strain regime, we found statistically significant changes in the relative expression of mRNAs for a total of 15 genes, suggesting a role for mechanical signalling in their regulation. Among these were seven multifunctional interleukins, in which alterations in gene expression ranged from a 2.11-fold downregulation of *IL1A* at 12 h, to a 3.47-fold upregulation of *IL8* at 24 h (Fig. 1).

Eight genes representing other cytokine and growth factor families showed comparable mechanical sensitivity (Fig. 2). Of these, *FIGF/VEGFD* and *OPG/TNFSF11B* were downregulated, while *PDGFA*, *INHBA*, *GDF8* and two transforming growth factor-β genes, *TGFB1* and *TGFB3*, were upregulated at various points in the time scale. Only two genes, *CSF2/GMCSF* and *IL11*, were found to be consistently upregulated across all three time points. A further six genes (*IFNA5*, *IFNB1*, *IL18*, *TGFBR2*, *TNFSF10* and *TNFSF13*) all showed alterations in expression of twofold or greater, but none reached statistical significance (Table 1).

Discussion

The study of the response of PDL cells to mechanical deformation in terms of interacting cytokine networks has been limited until recently by an inability to assay multiple genes concurrently. The fusion of real-time RT-PCR with microarray technology, enabling the simultaneous assay of a large panel of genes using relatively small quantities of RNA, now provides an opportunity to understand cytokine networks and how these regulate complex biological processes, such as tooth movement. An advantage of the study design is that by measuring control and experimental samples in triplicate, it is possible to obtain statistically robust and therefore more meaningful data. The custom to date has been to express data in terms of fold-change with a treated over control ratio (T/C) of ±2 being regarded as representing a significant change in expression. Our findings suggest that this will both under- and over-report the number of genes influenced by the applied force. For the majority of genes in which we measured a statistically significant change, the T/C ratio was less than 2, and for several others in which the T/C ratio was greater than 2, the difference did not reach statistical significance.

A surprising absentee from the interleukin family members to show a significant change in mRNA expression

Table 1. Alterations in gene expression by cultured human periodontal ligament cells following intermittent tensile mechanical strain

		Fold up- or downregulation (experimental/control)					
Name of gene	Description	6 h	*p*-value	12 h	*p*-value	24 h	*p*-value
CSF1	Colony stimulating factor 1 (M-CSF)	1.18	0.4191	−1.01	0.9268	1.14	0.2422
CSF2	Colony stimulating factor 2 (GM-CSF)	1.91†	0.0288†	1.56†	0.0058†	2.45†	0.0136†
CSF3	Colony stimulating factor 3	ND	ND	ND	ND	ND	ND
FAM3B	Family with sequence similarity 3, member B	ND	ND	ND	ND	ND	ND
FASLG	Fas ligand (TNF superfamily, member 6)	ND	ND	ND	ND	ND	ND
	C-fos induced growth factor (VEGF D)	−2.24*	0.0566	−1.97†	0.0355†	−1.43	0.0818
GDF10	Growth differentiation factor 10	ND	ND	ND	ND	ND	ND
GDF11	Growth differentiation factor 11	−1.09	0.3915	1.17	0.6197	1.09	0.3151
GDF2	Growth differentiation factor 2	ND	ND	ND	ND	ND	ND
GDF3	Growth differentiation factor 3	ND	ND	ND	ND	ND	ND
GDF5	Growth differentiation factor 5	1.25	0.2926	1.38	0.4268	1.17	0.2600
GDF8	Growth differentiation factor 8	1.21	0.5523	2.03†	0.0185†	1.14	0.5204
GDF9	Growth differentiation factor 9	−1.20	0.6926	−1.37	0.3527	−1.57	0.2086
IFNA1	Interferon-α1	−1.17	0.5467	1.25	0.5743	1.49	0.4072
IFNA2	Interferon-α2	ND	ND	ND	ND	ND	ND
IFNA4	Interferon-α4	ND	ND	ND	ND	ND	ND
IFNA5	Interferon-α5	1.61	0.2962	ND	ND	*2.99	0.1861
IFNA8	Interferon-α8	ND	ND	ND	ND	ND	ND
IFNB1	Interferon-β1, fibroblast	2.47*	0.3497	−2.31*	0.4131	ND	ND
IFNG	Interferon-γ	ND	ND	ND	ND	ND	ND
IFNK	Interferon-κ	ND	ND	ND	ND	ND	ND
IL10	Interleukin-10	ND	ND	ND	ND	ND	ND
IL11	Interleukin-11	1.58†	0.0248†	2.12†	0.0212†	1.84†	0.0030†
IL12A	Interleukin-12A	1.26†	0.0133†	1.80	0.0542	1.41†	0.0144†
IL12B	Interleukin-12B	−1.50	0.2009	−1.33	0.2585	1.11	0.5731
IL13	Interleukin-13	−1.97	0.1637	1.91	0.4221	ND	ND
TXLNA	Taxilin-α	1.14	0.5446	1.48	0.2089	1.31	0.0868
IL15	Interleukin-15	1.23	0.6537	1.01	0.9874	−1.30	0.3769
IL16	Interleukin-16	−1.23	0.2314	−1.01	0.9763	1.13	0.4631
IL17	Interleukin-17	ND	ND	ND	ND	ND	ND
IL17B	Interleukin-17B	ND	ND	ND	ND	ND	ND
IL17C	Interleukin-17C	−1.67	0.5156	−1.21	0.8481	−1.04	0.7933
IL17E	Interleukin-17E	ND	ND	ND	ND	ND	ND
IL18	Interleukin-18	1.14	0.5790	1.01	0.9833	2.20*	0.1738
IL19	Interleukin-19	ND	ND	ND	ND	ND	ND
IL1A	Interleukin-1α	−1.49	0.1785	–2.11†	0.0026†	–1.76	0.0877
IL1B	Interleukin-1β	1.22	0.5931	−1.69	0.5803	1.04	0.9476
IL1F10	Interleukin-1 family, member 10	ND	ND	ND	ND	ND	ND
IL1F5	Interleukin-1 family, member 5	ND	ND	ND	ND	ND	ND
IL1F6	Interleukin-1 family, member 6	ND	ND	ND	ND	ND	ND
IL1F7	Interleukin-1 family, member 7	1.15	0.6835	−1.08	0.8644	−1.50†	0.0104†
IL1F8	Interleukin-1 family, member 8	ND	ND	ND	ND	ND	ND
IL1F9	Interleukin-1 family, member 9	ND	ND	ND	ND	ND	ND
IL2	Interleukin-2	ND	ND	ND	ND	ND	ND
IL20	Interleukin-20	ND	ND	ND	ND	ND	ND
IL21	Interleukin-21	ND	ND	ND	ND	ND	ND
IL22	Interleukin-22	ND	ND	ND	ND	ND	ND
IL24	Interleukin-24	ND	ND	ND	ND	ND	ND
IL3	Interleukin-3 (colony-stimulating factor, multiple)	ND	ND	ND	ND	ND	ND
IL4	Interleukin-4	ND	ND	ND	ND	ND	ND
IL5	Interleukin-5 (colony-stimulating factor, eosinophil)	ND	ND	ND	ND	−1.56	0.0925
IL6	Interleukin-6 (interferon β2)	−1.25†	0.0427†	−1.02	0.9029	−1.03	0.8168
IL7	Interleukin-7	−1.49†	0.0475†	–1.02	0.9331	−1.31	0.1573
IL8	Interleukin-8	1.21	0.1806	1.29	0.1345	3.47†	0.0032†
IL9	Interleukin-9	ND	ND	ND	ND	ND	ND
INHA	Inhibin-α	1.23	0.8340	−1.03	0.9677	1.08	0.7553
INHBA	Inhibin-βA (activin A)	1.18	0.2485	1.51	0.1270	1.25†	0.0183†
LEFTY2	Left-right determination factor 2	ND	ND	ND	ND	ND	ND
LTA	Lymphotoxin-α	ND	ND	ND	ND	ND	ND
LTB	Lymphotoxin-β	ND	ND	ND	ND	ND	ND

Table 1. Continued

Name of gene	Description	Fold up- or downregulation (experimental/control)					
		6 h	*p*-value	12 h	*p*-value	24 h	*p*-value
NODAL	Nodal homologue (mouse)	ND	ND	ND	ND	ND	ND
PDGFA	Platelet-derived growth factor α polypeptide	1.09	0.7874	1.68	0.2603	1.73†	0.0490†
TGFA	Transforming growth factor-α	ND	ND	ND	ND	1.31	0.4643
TGFB1	Transforming growth factor-β1	−1.02	0.9025	1.68	0.3080	1.54†	0.0404†
TGFB2	Transforming growth factor-β2	1.20	0.1123	1.24	0.2499	1.01	0.9238
TGFB3	Transforming growth factor-β3	1.64	0.2297	4.02†	0.0285†	3.06*	0.0733
TGFBR1	Transforming growth factor-β receptor I	−1.04	0.8921	1.06	0.8159	1.27	0.3857
TGFBR2	Transforming growth factor-β receptor I	1.52	0.6623	−1.39	0.4019	−2.58*	0.1027
TNF	Tumor necrosis factor (TNF)	ND	ND	ND	ND	ND	ND
OPG	TNF receptor superfamily, member 11b	−1.17†	0.0256†	1.06	0.7117	−1.06	0.6950
TNFSF10	TNF (ligand) superfamily, member 10	−1.72	0.6435	−4.14*	0.1672	−3.39*	0.0639
RANKL	TNF (ligand) superfamily, member 11	ND	ND	ND	ND	ND	ND
TNFSF12	TNF (ligand) superfamily, member 12	−1.06	0.5944	1.08	0.7679	− 1.11	0.5566
TNFSF13	TNF (ligand) superfamily, member 13	1.36	0.4491	3.28*	0.0815	2.35*	0.0725
TNFSF13B	TNF (ligand) superfamily, member 13b	−1.02	0.9164	1.34	0.3546	−1.05	0.9179
TNFSF14	TNF (ligand) superfamily, member 14	ND	ND	ND	ND	ND	ND
TNFSF4	TNF (ligand) superfamily, member 4	1.69	0.2899	1.86	0.2345	1.63	0.3659
TNFSF7	TNF (ligand) superfamily, member 7	ND	ND	ND	ND	ND	ND
TNFSF8	TNF (ligand) superfamily, member 8	ND	ND	ND	ND	ND	ND
CALIBRATOR GENES							
B2M	β_2-Microglobulin	−1.03	0.8209	1.09	0.3925	1.24	0.0547
HPRT1	Hypoxanthine phosphoribosyltransferase 1	−1.13	0.5397	−1.43	0.3537	−1.18	0.1813
RPL13A	Ribosomal protein L13A	−1.00	0.9869	1.09	0.4178	−1.14	0.3034
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	−1.01	0.9474	1.09	0.4331	1.10	0.1894
ACTB	β-Actin	1.17	0.5023	1.11	0.5822	−1.00	0.9652

†Statistically significant change, $p < 0.05$; *experimental/control ratio greater than ± 2 but not statistically significant at $p < 0.05$. ND, not detected.

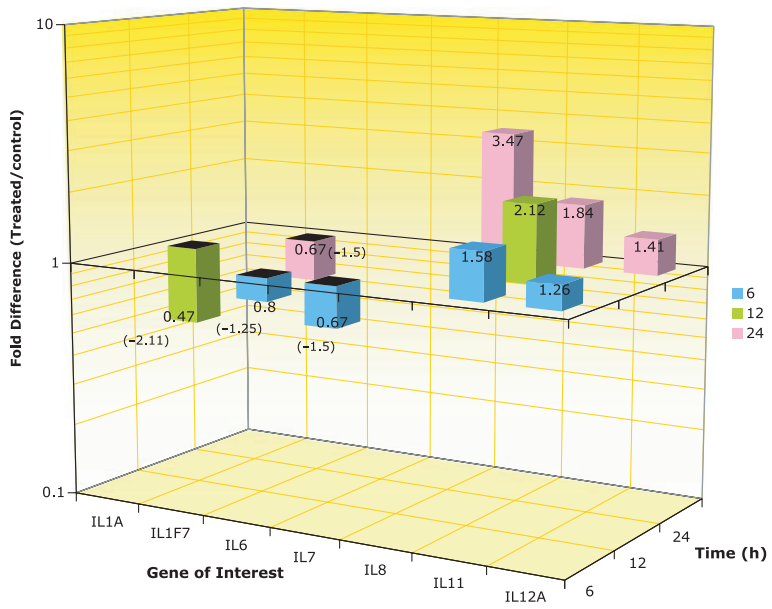

Fig. 1. Three-dimensional histogram of the seven interleukin family members that showed statistically significant ($p < 0.05$) changes in mRNA expression in response to intermittent tensile mechanical strain. The logarithmic *y*-axis scale allows for graphical representation of positive and negative regulatory events, relative to the point of no effect ($2^{-\Delta\Delta CT} = 1$). Negative values appearing in parentheses correspond to those reported in Table 1 and have been reported as negative reciprocals to highlight downregulatory events.

was the *IL1B* gene, although *IL1A* expression was found to be significantly downregulated. Interleukin-1β has been widely investigated in cultured PDL cells and reported to be both stimulated (2,3) and inhibited (4,5) by mechanical deformation. Isoforms of IL-1 are potent bone-resorbing agents (29–31), and two other genes encoding cytokines capable of stimulating bone resorption, *IL6* (32) and *IL7* (33,34), were also downregulated. The non-responsiveness and/or downregulation of these genes is consistent with sites of tensile strain in the PDL being characterized by osteogenesis, as was the stimulation of the message for IL-12A, a cytokine with a broad range of biological activities, including inhibition of osteoclast formation and bone resorption in synergy with IL-18 (35,36). In these circumstances, it is therefore not surprising that *RANKL* was not expressed, or that *OPG* expression was slightly inhibited.

Two genes that were consistently upregulated across all three time points

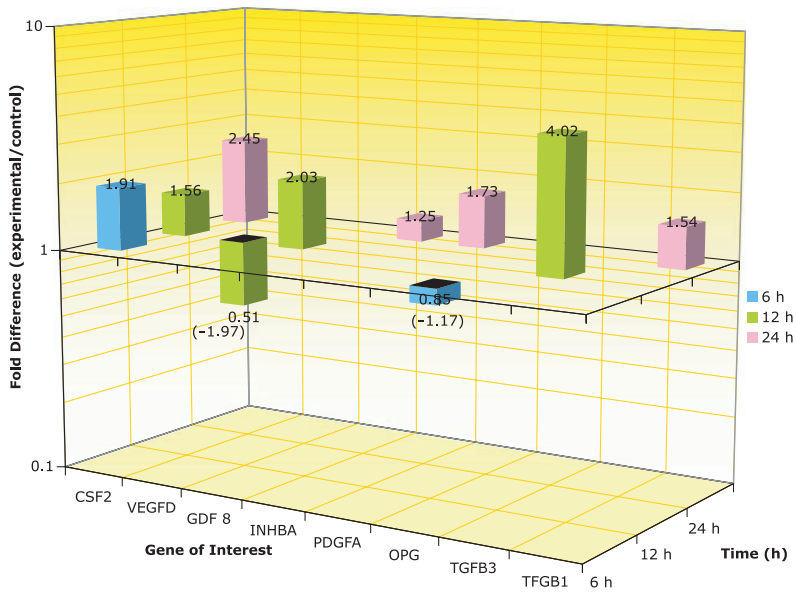

Fig. 2. Three-dimensional histogram of eight genes representing a diverse array of growth factor and cytokine families. All showed statistically significant ($p < 0.05$) regulatory changes in response to the experimentally applied strain regimen. The logarithmic *y*-axis scale allows for graphical representation of positive and negative regulatory events, relative to the point of no effect ($2^{-\Delta\Delta CT} = 1$). The negative values in parentheses correspond to those reported in Table 1. These are negative reciprocals of regulatory fold-differences of less than one, reported as such to simplify identification.

were *IL11* and *CSF2*/*GMCSF*. Interleukin-11 is a multifunctional cytokine produced by certain cells of the mesenchymal lineage, including fibroblasts, bone marrow stromal cells and articular chondrocytes (37). Like IL-6, with which it shares a common receptor (gp130), IL-11 can stimulate bone resorption (38), but has also been shown to have anabolic effects on bone. Interleukin-11 alone, or in combination with bone morphogenetic protein-2 (BMP-2), induces osteoblastic differentiation in mouse mesenchymal cells (39), suggesting a role for this cytokine in bone formation. In a related study, in which the expression of a panel of osteogenesis-related genes by PDL cells was evaluated, we found that the bone morphogenetic proteins *BMP2* and *BMP6* were among the genes significantly upregulated by cyclic tensile strain (MN Pinkerton, DC Westcott, BJ Gaffey, KT Beggs, TJ Milne, MC Meikle, unpublished findings). It has also been reported that both IL-11 and BMP-2 expression are stimulated in human PDL cells by TGF-β (40).

The upregulation of the *CSF2*/*GMCSF* gene was perhaps our most unexpected finding. Granulocyte macrophase-colony stimulating factor shows biphasic actions in regulating osteoclast differentiation and function and can act as both a stimulator (41) and as an inhibitor of bone resorption (42–44). The literature suggests that the inhibitory or stimulatory effects of granulocyte macrophase - colony stimulating factor (GM-CSF) are facilitated through several regulatory pathways: inhibition of osteoclast differentiation through direct action on osteoclast precursors (45); downregulation of TNF receptors 1 and 2 on the surface of osteoclast progenitor cells (46); or indirectly, through the up- or downregulation of macrophage colony stimulating factor (M-CSF) secretion by osteoblasts and stromal cells (47–49). The specificity of the regulatory response is likely to be dependent upon the stage of differentiation of target cells and the particular model system being studied, as well as the temporal patterns of expression of interacting signalling molecules, and highlights the significance of cytokine cross-talk, pleiotropy and redundancy in determining the biological response.

Consistent with the hypothesis that tensile strain would favour an osteogenic pattern of signalling, the genes for two TGF-β isoforms, *TGFB1* and *TGFB3*, were upregulated. Transforming growth factors-β play complex roles in bone metabolism. Transforming growth factor-β_1 supports osteogenesis through its effects on both osteoblast and osteoclast cell populations, and *in vitro* studies suggest that TGF-β_1 is involved in the recruitment and proliferation of osteoblast progenitors, the early phases of osteoblast differentiation and the attenuation of osteoblast apoptosis (50). The biphasic regulatory effects of TGF-β_1 on osteoclasts are concentration dependent; at low concentrations TGF-β_1 supports osteoclast differentiation through alteration of the OPG/RANKL ratio, while at high concentrations differentiation is suppressed through multiple interacting pathways, not all dependent on RANKL/OPG or M-CSF expression (52,52). While the effects of TGF-β_3 on bone are less well documented, data to date suggest that TGF-β_3 has similar effects to the other isoforms and binds to the same receptor. Transforming growth factor-β_3 has been shown to be a potent regulator of osteoblast function and reported to be three-to-five times more potent than TGF-β_1 (53).

Of the other genes that were significantly altered by mechanical deformation, *VEGFD* (down) and *IL8* (up) are of particular interest. The periodontal tissues are highly vascularized, and new blood vessel formation or angiogenesis is a characteristic feature of the PDL response to tooth movement. Vascular endothelial growth factors are potent angiogenic factors and exist in four isoforms (A–D); the downregulation of *VEGFD* was therefore a surprise, particularly since we have found that *VEGFA* (but not *VEGFB*) was stimulated by cyclic tensile strain (unpublished findings). The *IL8* gene, in contrast, was upregulated 3.47-fold; the highest interleukin in the study. Interleukin-8 is a cysteine-x-cysteine (where x = non-specified amino acid)

*chemo*tactic cyto*kine* or chemokine, a large family of cell-to-cell signalling molecules that are major regulators of angiogenesis (54,55). The likely net effect of these changes is to promote blood vessel formation at tension sites.

It is not clear what precise role each of the three other genes that were upregulated by cyclic tension, *GDF8*, *INHBA* and *PDGFA*, might play in modulating the tissue response. However, it is likely to be related to osteogenesis. The *GDF8* (myostatin) gene is a negative regulator of skeletal muscle growth, and mice lacking *GDF8* show increased muscle mass and bone density compared with wild-type control mice. *In vitro* studies have shown that loss of *GDF8* function increases the differentiation of bone marrow-derived mesenchymal stem cells into osteoblasts, although the addition of recombinant *GDF8* was found not to significantly reduce the osteogenic response (56). The *INHBA* gene encodes activin-βA, another member of the TGF-β superfamily. Activins play fundamental roles in cell differentiation and development and have been shown to modulate the biological effects of insulin-like growth factor, a growth factor known to influence the differentiation of committed osteoblast precursor cells (57). Platelet-derived growth factors also influence the differentiation of committed osteoblast precursors and have been shown in addition to stimulate the migration and proliferation of connective tissue cells, including fibroblasts (58), endothelial cells (59) and osteoblasts (60,61).

The demonstration that cytokines (frequently referred to in the literature as inflammatory mediators or *proinflammatory* cytokines) play an important role in cell-to-cell signalling in the PDL has led to the concept that orthodontic tooth movement is an inflammatory process (1,4,9,10). Apart from the fact that tooth movement does not meet the four classical criteria for inflammation described by Celsus, except perhaps for the pain, if this were true, one might expect to find mRNAs for those cytokines described in OMIM (Online Mendelian Inheritance in Man, www.ncbi.niu.nih.gov/sites/entrez?db=OMIM) as immunoregulatory lymphokines. This proved not to be the case. We could find no evidence for the expression of *IL2–IL5*, *IL17* or *IL17B* at CT values less then 35 in PDL cells. The majority of the cytokine genes found to be expressed and responsive to mechanical deformation were in fact *pleiotropic* cytokines, such as *IL1A*, *IL6*, *IL11* and *IL12A*, which have multiple biological activities. Describing orthodontic tooth movement as an inflammatory process creates the impression that it is a pathological event. For the most part it is not, unless the clinician or animal investigator happens to be applying excessive force to the teeth, causing tissue destruction. The tissue response to tooth movement is best regarded as an exaggerated form of physiological turnover combined with foci of tissue repair, particularly at compression sites where hyalinized tissue, adjacent bone and cementum are undergoing resorptive remodelling.

In conclusion, this investigation has expanded our knowledge of the cytokines and growth factors expressed by human PDL cells in culture and identified several new mechanoresponsive genes. However, additional studies are necessary to establish whether the expressed genes are translated into protein and, if they are, whether the proteins are biologically active. This will require: (1) assaying culture supernatants for the expressed proteins of interest by ELISAs and testing their biological activity with the appropriate bioassays; followed by (2) immunolocalization of the proteins *in situ* with specific antibodies using animal models of tooth movement. A more complete understanding of cytokine biology will depend upon recognizing the artifactual nature of *in vitro* experimental systems, the ability to reconcile *in vitro* experimental data with those from appropriate *in vivo* models, and appreciation of cytokine biology in terms of complex interacting networks, rather than just individual mediators acting in isolation.

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