ORIGINAL ARTICLE

Orthodontic tooth movement and de novo synthesis of proinflammatory cytokines

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Interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) are proinflammatory cytokines that are thought to play a role in bone remodeling, bone resorption, and new bone deposition. In the present work, in situ hybridization was performed to measure the messenger RNA expression of IL-1β, IL-6, and TNF-α at 3, 7, and 10 days after the application of orthodontic force on the maxillary first molars of 12 rats. The contralateral side and 3 untreated rats served as controls. Measurements of the messenger RNA expression were selected as the means to investigate the role of orthodontic force in de novo synthesis of proinflammatory cytokines. After the application of force, the induction of IL-1β and IL-6 was observed to reach a maximum on day 3 and to decline thereafter. No messenger RNA induction of either cytokine was measured in the control teeth. The messenger RNA expression of TNF-α was not detected at any time point of this study in the experimental or contralateral sides or in the control animals. Our data support the hypothesis that these proinflammatory cytokines may play important roles in bone resorption after the application of orthodontic force. (Am J Orthod Dentofacial Orthop 2001;119:307-12)

The transference of orthodontic forces to the periodontal tissue involves several biologic mechanisms and ultimately results in excessive bone[1] and root resorption.[2-6] The relationship between this degradation activity and force is not clear.[7] However, it has been postulated that systemic factors may be involved in the regulation of the tissue-degrading activity.[8] Some studies have suggested roles for the immune system in the regulation of bone remodeling through cytokine production by inflammatory cells that migrated from dilated periodontal ligament (PDL) capillaries after the application of orthodontic force.[9] Cytokines are proteins that act as signals between the cells of the immune system. They are produced during the activation of immune cells and usually act locally, but some cytokines act systemically with overlapping functions. Previous studies have implicated certain cytokines in bone remodeling in vitro and in vivo, including interleukin-1 (IL-1),[8] interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α).[10,11] These cytokines each have multiple activities,[12] which include bone remodeling, bone resorption, and new bone deposition.[13,14] IL-1 is a key mediator in a variety of activities in immune- and acute-phase inflammatory responses.[15] It has been found that IL-1β increases in human gingival fibroblasts during orthodontic movement.[16] IL-6 regulates immune responses in inflammation sites,[17] and it has an autocrine/paracrine activity that stimulates osteoclast formation and the bone-resorbing activity of preformed osteoclasts.[18,19] IL-1β and TNF-α have been implicated in the process of bone remodeling.[20] On activation, monocytes and macrophages synthesize and release IL-TNF-α, which is released by monocytes after the activation of leukocyte cultures in vitro with a mitogen or an antigen, and is a potent inducer of bone resorption in fetal rat and mouse organ cultures.[20] In addition, TNF-α levels are elevated during orthodontic tooth movement in the human gingival sulcus. The source may be adjacent gingiva but is more likely the compressed PDL and resorbing bone adjacent to the root surface.[21] In the present study, we examined whether orthodontic movement induces gene transcription of IL-1β, IL-6, and TNF-α in the PDL of rats.

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MATERIAL AND METHODS

Animals

Fifteen Wistar male rats (weight, 180 ± 3.2 g) that were 40 to 45 days old were used in this study.[6] The animals were fed a standard pellet diet with tap water ad libitum (801157 W Expanded Pellets; Stepfield Witham, Essex, UK). The rats were divided into 3 groups of 4 animals each; 3 untreated rats served as control. In the experimental animals, the maxillary

Table I. Survey of cytokine probes used

Probe	*Exon*	*GenBank accession no.*	*Complementary to bases*
Rat IL-1β	Exon 1	M98820	639-686
	Exon 2		569-616
	Exon 3		278-325
	Exon 4		295-342
Rat IL-6	Probe 2	M26744	139-187
	Probe 3		180-223
Rat TNF-α	Exon 1	00475	913-960
	Exon 2		2059-2106
	Exon 3		2152-2199
	Exon 4		2316-2363

right first molars were moved mesially by means of a closed coil spring (Elgiloy spring, F-0.008x0.032; Rocky Mountain Dental Products, Denver, Colo) and ligated to the mesial aspect the of the first molar and through the eyelet on an incisor band. The strain applied was 500 mN. There was no reactivation during the experimental period. The maxillary left molars served as controls. The animal's weight was recorded on the day of operation (day 0) and before death. All operations were performed with general anesthetic (subcutaneous injection of Dormicum/Hypnorm solution; dosage, 0.15-0.2 mL/100 g body weight; Roche, Basel, Switzerland). The experimental periods were 3, 7, and 10 days, respectively. On the day of death, the rats received an overdose of anesthetic and were perfused through the left heart ventricle with 4% paraformaldehyde with 0.2% picric acid solution. After perfusion, the right and the left halves of the maxillae, including first, second, and third molars were dissected and placed in 4% paraformaldehyde with 0.2% picric acid for 24 hours at 4°C, then rinsed in 0.1 mol/L phosphate buffer, pH 7.4. The maxillae were then decalcified in a 10% EDTA solution for 10 days, immersed in 30% sucrose/phosphate-buffered saline solution over night, and mounted in Tissue Tek (OCT compound, embedding medium; SAKURA, Zoeterwoude, The Netherlands). Specimens were frozen in dry ice and kept in –70°C until used. Parasagittal sections of the mesiodistal aspect of the teeth were cut in 5- to 6-μ–thick frozen sections at –21°C. Sections were collected on SuperFrust/Plus slides (Menzel-Glaser, Braunschweig, Germany). The area of investigation was the compressed zone of the distal, middle, or mesial root. Every third glass (4 sections in each glass) was chosen for labeling of IL-1β, IL-6, and TNF-α. From each specimen, the glass that contained sections in which the hyalinized zone was longest in the coronal-apical direction was chosen as a basis for the investigation. The number of labeled cells for each of the cytokines investigated was counted per 100 mm^2 tissue section. The tissue section areas were measured by image analysis (Seescan-Image Analysis System, Cambridge, UK).

In situ hybridization

In situ hybridization of the tissue sections was performed as described elsewhere.[22] In brief, cryostat sections were placed on glass slides (ProbeOn precleaned microscope slides; Fischer Scientific, Pittsburgh, Pa). The sections were dried at 56°C for 10 minutes. The oligonucleotide sequences were obtained from GenBank (Los Alamos, NM), and antisense probes were designed with MacVector software (Oxford Molecular, Madison, Wis) (Table I). Synthetic oligonucleotide probes were labeled with deoxyadenosine-5′,-α-(thio)-triphosphate [^{35}S] (Dupont Scandinavia, Stockholm, Sweden) with terminal deoxynucleotidyl transferase (Amersham, Little Chalfont, UK). To increase the sensitivity of the method, a mixture of 4 different (approximately 48-bp–long) oligonucleotide probes was used. After hybridization, slides were rinsed 3 times for 15 minutes each time at 55°C in 1 × SSC (0.15 mol/L NaCl plus 0.015 mol/L sodium citrate), allowed to adjust to room temperature, dipped in distilled water, dehydrated through graded ethanol (60%, 70%, and 95%), and air dried. The slides were immersed in NTB2 emulsion (Eastman Kodak, Rochester, NY) and exposed at 4°C for 15 days. After development in D19 (Eastman Kodak), the slides were stained with cresyl violet and mounted with Entellan (Merck, Darmstadt, Germany). Coded slides were examined by dark field microscopy at ×10 magnification. Cells that expressed more than 15 grains with a star-like distribution over their cytoplasm were judged as positive. In cells judged negative, the number of grains was usually 0 to 2 per cell, and the grains were randomly scattered over the cell and not distributed in a star-like fashion. The cellular distribution was always checked under light microscopy at ×20 magnification. The variability of the hybridization procedure was ±15%. Control sense oligonucleotide probes used in parallel with the cytokine antisense probes on tissue produced uniformly weak background signals without revealing any positive cells. As a positive control for the method, hybridization was performed with all examined probes in splenocytes stimulated with concavalin A, and high levels of messenger RNA (mRNA)-positive cells for the examined cytokines were detected.

Statistical analysis

Student *t* test (unpaired method) was used because the values were normally distributed. In all tests, a

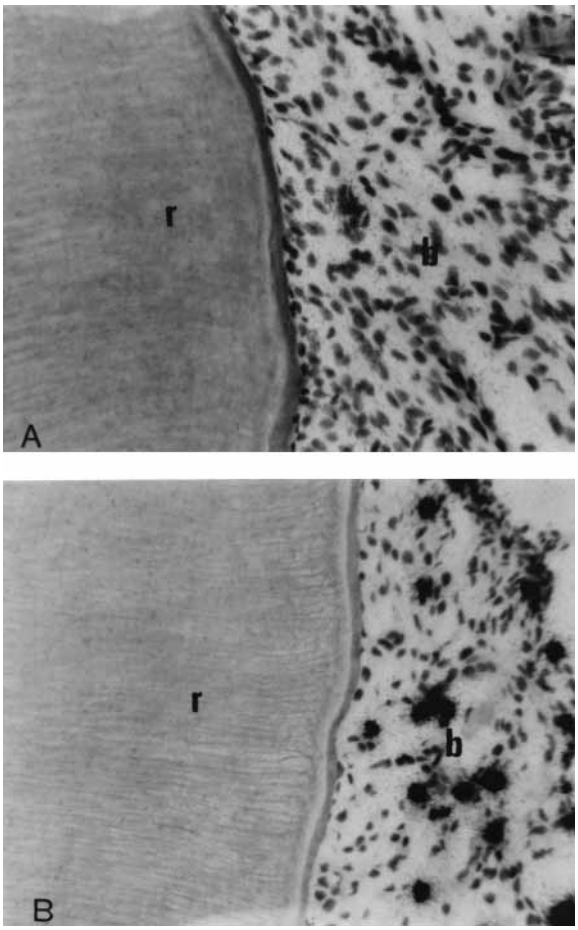

Fig 1. Mesial area of mesial root of maxillary first molar of rat. Cytokine mRNA expression. In this example, IL-1β mRNA was detected by in situ hybridization. **A**, Contralateral control side with no induction of IL-1β mRNA; **B**, hybridizing cells in bright field microscopy at ×200 magnification on compression side after tooth movement for 3 days. *r*, Root; *b*, alveolar bone.

probability value of less than .05 was defined as the level of significance. All tests of significance were 2 sided. For each cytokine, 2 fields from the experimental and 2 fields from the control first maxillary molar were used for evaluation. The labeled cells were counted and divided by the number of evaluated sections for each tooth to obtain the mean value.

RESULTS

The number of cytokine mRNA expressing cells for IL-1β, IL-6, and TNF-α was determined with the use of in situ hybridization with radiolabeled oligonucleotide probes. The number of positive cells observed in this period at 3 days was significantly greater on the compression side compared with the control side. A statisti-

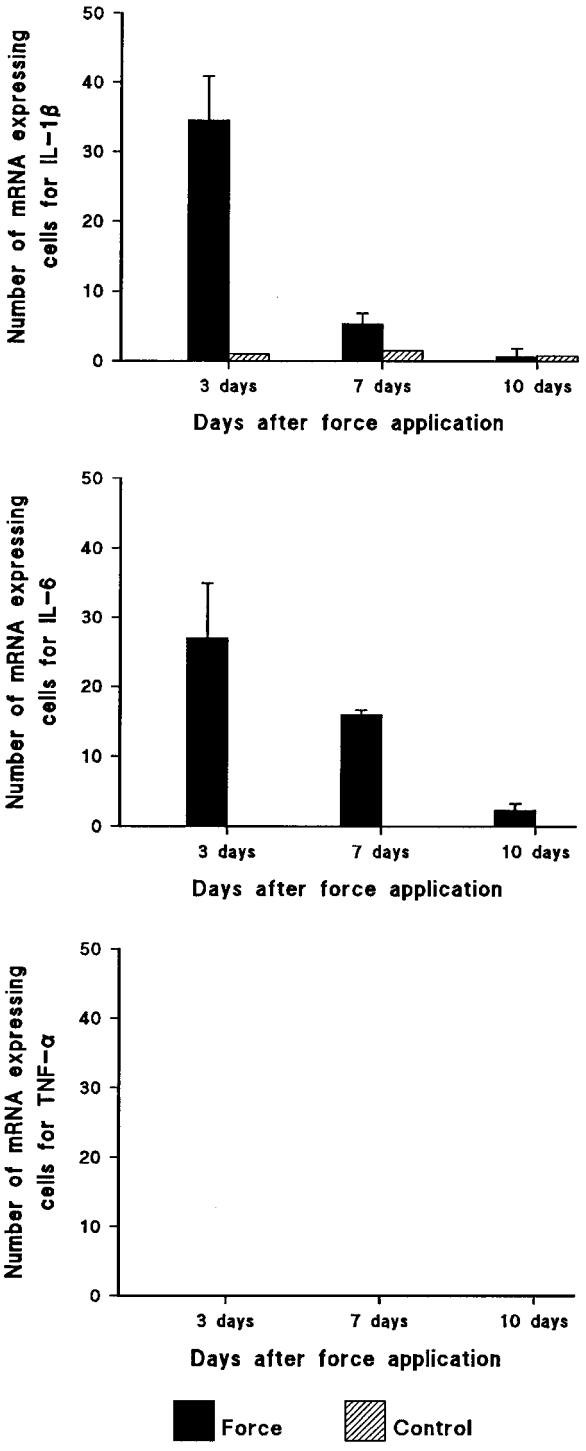

Fig 2. Numbers of cells that express mRNA of IL-1β, IL-6, and TNF-α as detected by in situ hybridization technique. Sections were prepared from 4 rats in each group. For each rat, quadruplicate sections were made. Means and SD of 4 rats per group are shown.

cally significant difference in cytokine mRNA levels ($P < .05$) was observed between the experimental pressure side and both the contralateral control side and the control rats. After the application of force, maximum induction of IL-1β and IL-6 was observed on day 3 of orthodontic tooth movement on the compression side and in the PDL space. IL-1β and IL-6 expression was also detected in the bone marrow cells that faced the PDL (Fig 1). On day 7, expression had decreased, and the levels became undetectable on day 10 (Figs 1 and 2). The control side demonstrated a slight induction of mRNA for IL-1β and no induction of mRNA for IL-6. However, there was no cytokine expression in the untouched control group of rats. To confirm the specificity, sense control was used for each cytokine, and no positive cells were detected. mRNA expression of TNF-α was not detected at any time during the 10 days of this study in the experimental, contralateral, and untreated control rats. The decalcifying protocol used in this study achieved an optimal balance between hybridized mRNA and the preservation of tissue morphologic factors. The weight of the animals was not affected by the treatment.

DISCUSSION

This work showed increased levels of IL-1β and IL-6 mRNA, but not TNF-α mRNA, in response to orthodontic tooth movement. Although previous studies showed elevated levels of all these proinflammatory cytokines in human gingival crevicular fluid,[11,23,24] it was not clear whether the induction of this cytokine was generated at transcription or at translation or was simply a product of the application of force. Therefore, our approach to study the mRNA expression of these cytokines was selected to investigate the effect of orthodontic force on the mRNA expression of these cytokines. Studies of the role of cytokines have focused mainly on the determination of cytokine levels in body fluids. The principle used here for the detection of cytokines (an in situ hybridization technique with subsequent counting of cytokine mRNA-expressing cells) enabled us to study the cellular induction of cytokines, which act autocrinely or paracrinely with a very short half-life and have a high affinity for nearby receptors. IL-1 exists in 2 forms, alpha and beta,[25,15] of which IL-1β is the one mainly involved in bone metabolism, stimulation of bone resorption,[26,27] and inhibition of bone formation.[28] IL-1β plays a central role in the inflammatory process. Previous studies in cats have detected prostaglandin E and IL-1β in the periodontium of teeth undergoing movement.[29] The staining of cat PDL cells for IL-1β showed the presence of bound signal complexes in the plasma membrane, which was expected because it has been shown that receptors for IL-1β are present on fibroblasts.[30] The potential sources of IL-1β during tooth movement include cells such as fibroblasts, macrophages, cementoblasts, cementoclasts, osteoblasts, and osteoclasts. In the early stages of tooth movement (at 12 and 24 hours), many PDL cell types stained positively for IL-1β.[9] The experimental tooth movement leads to significantly increased recruitment of cells that belong to the mononuclear phagocytic system, and it was suggested that the presence of neuroimmune interactions may be of importance in the initial inflammatory response and the regenerative processes of the PDLs that are incident to orthodontic tooth movement.[31] The macrophage has the ability to produce cytokines, such as IL-1β and IL-6, the levels of which are known to increase during orthodontic tooth movement.[29] IL-1β may act synergistically with TNF-α[15] and be a powerful inducer of IL-6.[32,33] IL-1β, IL-6, and TNF-α were suggested to stimulate bone resorption and bone-cell replication.[20,34]

Our findings on mRNA levels of IL-1β are consistent with previously reported data on increased levels of these cytokines in human gingival crevicular fluid.[11,21,35] However, the situation was different for TNF-α, because no measurable mRNA was recorded in the PDL. This finding may be explained by the following: (1) This cytokine is mainly released during the application of force and not produced de novo. (2) TNF-α may be regulated at the transcription level, and although protein levels are still measurable in body fluids, the mRNA message is downregulated. Cytokines are tightly regulated during induction, gene transcription and translation, protein synthesis, and secretion. Once released, the cytokines are regulated in the circulation and at the receptor level on target cells.[36] Therefore, the discrepancy between the absence of TNF-α mRNA expression observed in this study versus the amount detected in a previous study[21] may be due to a negative signal during the process of gene transcription, which may be the result of a feedback mechanism caused by increased TNF-α protein levels. This was considered to be a probable explanation, because the expression of the cytokines is steadily controlled in the producing cells and is 1 of the most important regulatory steps in this control is gene transcription. Several positive and negative transcription factors function in a concerted manner to regulate transcription of cytokines at the promoter or intron region. It is also common for the transcription of cytokine genes to initiate immediately on activation and to shut down quickly, even in the continuous presence of the stimulating agent.[37] Furthermore, the contradiction between previous reports in which TNF-α was demonstrated in gingival crevicular fluids of human orthodontic patients and our study in rats in which TNF-α mRNA was not detected could be due to species-related differences.

Finally, the results of this study support the hypothesis that proinflammatory cytokines play a potent role in bone resorption after the application of orthodontic force. In our experiment, the maximal level was detected on day 3 after the application of orthodontic force. The decreased number of IL-1β–positive and IL-6–positive cells on days 7 and 10 accounts for the return of the cytokines to baseline levels. The spring did not require reactivation during the experiment. This fact may explain the reason that IL-1β and IL-6 levels were decreased at 7 and 10 days.

CONCLUSION

The data of this work showed that the application of orthodontic force induces de novo synthesis of the proinflammatory cytokines IL-1β and IL-6, which support the hypothesis that these proinflammatory cytokines play important roles in bone resorption during the application of orthodontic force. Furthermore, our work has shown that in situ hybridization with complementary DNA probes to detect cytokine transcripts in frozen tissue sections and the subsequent counting of cytokine mRNA-expressing cells is a useful method for the assessment of cytokine gene expression during different immune challenges, such as orthodontic movement. It can lead to a better understanding of the molecular basis for cell-cell communication during mechanically induced remodeling and enable us to study further the involvement of other cytokines and chemokines, which may play a role in bone remodeling during orthodontic tooth movement.

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