Expression of bone-resorptive and regulatory cytokines in murine periapical inflammation

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Abstract

Periapical bone destruction earlier was shown to be mediated primarily by interleukin (IL)-1α in a rat model. The production and action of IL-1α is in turn potentially modulated by a network of cytokines, which are produced by infiltrating T-helper type 1 (Th1) and type 2 (Th2) lymphocytes, and resident connective tissue cells within the lesion. This study was designed to examine the kinetics of expression of 10 cytokines in experimentally induced murine periapical lesions, including bone-resorptive [IL-1α, tumour necrosis factor α (TNFα), IL-6, IL-11], Th1-type [IL-2, IL-12, interferon-γ (IFNγ)] and Th2-type (IL-4, IL-6, IL-10, IL-13) mediators. Cytokine mRNA expression was assessed qualitatively by reverse transcription-polymerase chain reaction, and cytokine proteins quantified by enzyme-linked immunosorbent assay. IL-1α and TNFα protein and mRNA were highly expressed, beginning on day 7, and increased to day 28. IL-6 increased to day 14 and then declined, whereas the expression of IL-11 was not modulated by pulp exposure. Most of the Th1-type cytokines, including IL-2, IL-12, and IFNγ, showed an increase in mRNA and/or protein expression in periapical lesions after pulpal exposure; the expression of Th2-type cytokines was similarly increased, but had declined at the latest time-point (day 28), suggesting possible inhibition by Th1-type mediators. Significant correlations were observed between levels of IL-1α and Th1-derived pro-inflammatory mediators IL-2, IL-12, TNFα, and IFNγ. There was a lack of correlation between IL-1α and Th2-type anti-inflammatory mediators, including IL-4, -6, and -10. These results indicate that a cytokine network is activated in the periapex in response to bacterial infection, and that Th1-modulated pro-inflammatory pathways may predominate during periapical bone destruction. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Cytokines; Bone-resorption; Inflammation; Periapical

1. Introduction

The periapical lesion represents an inflammatory and immune response against micro-organisms that invade and destroy the dental pulp. This response appears to be similar to that which occurs in response to bacterial infections elsewhere in the body, with the additional feature that alveolar bone surrounding the root apex is resorbed. A number of cytokines are able to induce bone resorption, including IL-1, TNF, IL-6 and IL-11. However, in previous studies in a rat model, IL-1α was strongly implicated as the primary stimulator of periapical bone destruction (Wang and Stashenko, 1993). Marked expression of IL-1α mRNA and protein was localized to infiltrating macrophages and polymorphonuclear leucocytes, as well as to connective tissue cells (Tani-Ishii et al., 1995; Wang et al., 1997). Extracts of periapical lesions possess bone-resorptive activity, which is mainly blocked by anti-IL-1α antibody (Wang and Stashenko, 1993).

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Administration of IL-1 receptor antagonist significantly inhibits expansion of the lesion (Stashenko et al., 1994).

Although IL-1α plays a central part in the pathogenesis of the peripical lesion, its production and activity are likely to be regulated by a network of other, primarily T-cell-derived, cytokines (Stashenko et al., 1998). Th1 cells are generally pro-inflammatory, express IFNγ and IL-2, and express and respond to IL-12 derived from macrophages (Seitz et al., 1996). IFNγ is a key mediator of macrophage activation and may, in combination with other stimuli such as lipopolysaccharide, upregulate IL-1 and TNF expression by this cell type (Collart et al., 1986; Gerrard et al., 1987; Seiling et al., 1994; Turner et al., 1989). TNFα induces the production of IL-1 (Kaushansky et al., 1988), and IL-1 stimulates its own synthesis in a positive feedback loop (Dinarello et al., 1987; Mauveil et al., 1988). These stimulatory pathways may accelerate bacterial infection-induced bone destruction. In contrast, cytokines produced by Th2 lymphocytes, including IL-4, -6, -10, and -13, downregulate the production of IL-1 (Fiorentino et al., 1991; Tilg et al., 1994; Horowitz and Lorenzo, 1996; Seitz et al., 1996; Yamamoto et al., 1997; Muchamuel et al., 1997), and inhibit the expression and activity of the Th1-type mediators (Mosmann and Coffman, 1989; Fiorentino et al., 1991). These IL-1 inhibitory functions may serve to protect periapical bone from destruction.

In the present study, the expression of bone-resorptive and regulatory cytokines was assessed in developing periapical lesions in the mouse, as a first step in characterizing the elements of the cytokine network that are key participants in modulating periapical bone destruction.

2. Materials and methods

2.1. Animals and induction of periapical lesions

Eleven-week-old C57Bl/6 male mice ($n = 61$) were obtained from Charles River Breeding Laboratory, Wilmington, MA and maintained in a conventional environment in the Forsyth Dental Center Animal Facility according to the guidelines of the institutional IACUC. For the induction of periapical lesions, mice were anaesthetized with ketamine HCl (62.5 mg/kg) and xylazine (12.5 mg/kg) in sterile phosphate-buffered saline by intraperitoneal injection, and mounted on a jaw-retraction board. All four first-molar pulps were exposed to the oral environment with a No. 1/4 round bur under a surgical microscope (Yu and Stashenko, 1987). The size of the exposure was equivalent to the diameter of the bur. In this model, all animals with pulp exposures develop intrapulpal infections by day 2,

as determined by positive cultures from extracted teeth (Tani-Ishii et al., 1994; P. Stashenko and L. Hou, unpublished observations). In addition, radiographically detectable periapical radiolucencies are present by day 14. Animals were killed at 7 ($n = 15$), 14 ($n = 15$) and 28 days ($n = 14$) after pulp exposure. Animals without exposures ($n = 17$) were used as controls (day 0). Samples from five animals per group were used for ELISA, and RT-PCR was done on the remainder.

2.2. Sample preparation

Periapical tissues surrounding the mesial and distal root apices were carefully extracted with surrounding bone in a block specimen under a surgical microscope. The gingiva, oral mucosa, tooth crown and bone marrow were dissected free of the samples and discarded. Periapical tissues were rinsed in phosphate-buffered saline, freed of clots, weighed and immediately frozen in dry ice/ethanol. Tissues were stored at $-70°C$ until protein and RNA extraction. Spleens obtained from mice immunized intraperitoneally (0.05 ml/g animal wt) with bovine serum albumin (fraction V; Sigma, St Louis, MO) in Freund's complete adjuvant (Sigma) were used as a positive control for all cytokines.

2.3. RNA extraction and cDNA synthesis

Tissue samples were ground in a precooled sterile mortar and pestle, and total cell RNA was obtained by guanidinium thiocyanate–phenol–chloroform extraction (RNAzolB; Tel-Test, Friendswood, TX). RNA precipitates were dissolved in diethyl pyrocarbonate-treated water containing RNase inhibitor (100 U/ml; Life Technologies, Gaithersburg, MD). The concentration of RNA was determined spectrophotometrically and samples were stored at $-70°C$. cDNA was constructed in a 20 μl reaction mix consisting of 3 μg of total cellular RNA, 1 μl of oligo dT primer (0.5 mg/ml; Life Technologies), 2 μl of 10 × PCR buffer with Mg^{2+} (Promega, Maiden, WI), 2 μl of 10 mM dNTP mix (Life Technologies) and 1 μl of MMLV RNaseH⁻ reverse transcriptase (200 U/μl Superscript II; Life Technologies). The reaction was incubated at 25°C for 10 min, followed by 42°C for 60 min and 95°C for 10 min to inactivate reverse transcriptase. cDNA was stored frozen at $-20°C$ until used in the PCR.

2.4. Polymerase chain reaction

The PCR primers (0.2 mg/ml; Genosys, Woodlands, TX) and expected product sizes for the 10 cytokines analysed are presented in Table 1. Primers were chosen from different exons so that any genomic DNA contamination would be easily detectable. PCR was done on 25 μl of a mixture which contained 1 μl of cDNA

Table 1
Primer sequences and size of products

IL-1α	sense	: GGCTCACTTCATGAGACTTG	
	antisense	: ACAAACTTCTGCCTGACGAG	(290bp)
IL-2	sense	: TGTTGATGGACCTACAGGAG	
	antisense	: CAGTTGCTGACTCATCATG	(294bp)
IL-4	sense	: TTTGAACGAGGTCACAGGAG	
	antisense	: TGCTCTTTAGGCTTTCCAGG	(293bp)
IL-6	sense	: CAACGATGATGCACTTGCAG	
	antisense	: GAAATTGGGGGTAGGAAGGAC	(276bp)
IL-10	sense	: TCAGCCACCTGAAGACTTTC	
	antisense	: TGTAGACACCTTGGTCTTGG	(324bp)
IL-11	sense	: TGGCCAGATAGAGTCGTTGC	
	antisense	: CATCAAGAGCTGTAAACGGC	(390bp)
IL-12	sense	: GGAGAAACAGTGAACCTCAC	
	antisense	: TTCTTTGCACCAGCCATGAG	(310bp)
IL-13	sense	: TGCCAAGATCTGTGTCTCTC	
	antisense	: GGATGGTCTCTCCTCATTAG	(336bp)
TNFα	sense	: CGCTCTTCTGTCTACTGAAC	
	antisense	: TGTCCCTTGAAGAGAACCTG	(301bp)
IFNγ	sense	: ACACTGCATCTTGGCTTTGC	
	antisense	: CTTGCTGTTGCTGAAGAAGG	(310bp)
β-actin	sense	: AGTACCCCATTGAACATGGC	
	antisense	: TCGGTCAGGATCTTCATGAG	(382bp)

from the reverse-transcription reaction, 0.5 μl of 10 mM dNTP mix, 1 μl sense primer, 1 μl antisense primer, 2.5 μl of 10 × PCR buffer with Mg^{2+}, 0.1 μl *Taq* polymerase (5 U/μl; Promega), and 18.9 μl autoclaved distilled water. Reactions were overlaid with 20 μl of mineral oil, and 35 cycles of PCR amplification were done according to the following cycling conditions: elongation, 72°C (2 min); melting, 93°C (1 min); and annealing, 60°C (IL-1α, IL-2, IL-11, IFNγ, β-actin), 55°C (IL-4, IL-6, IL-12, IL-13), or 52°C: (IL-10, TNFα). PCR products (15 μl) were analysed on 1.8% agarose gels and visualized by ethidium bromide staining. β-actin was amplified to assess the integrity of the RT-PCR. As a positive control, RT-PCR products for each cytokine were amplified from immunized spleen as shown in Fig. 1. PCR with RNA samples as template instead of cDNA served as negative controls.

2.5. Southern blotting

Southern hybridization was used to determine the authenticity of PCR products. Products were transferred from agarose gels to positively charged nylon membranes (Boehringer Mannheim, Indianapolis, IN). cDNA probes for IL-1α, -2, -4, -6, -10, -12, -13, TNFα, IFNγ (ATCC, Rockville, MD), and IL-11 (Genetics Institute, Cambridge, MA) were labelled with digoxygenin using a commercially available kit (hexanucleotide mix, 10 × dNTP labelling mix, Klenow enzyme; Boehringer Mannheim). Hybridization was detected by a chemiluminescent method consisting of antidigoxi-

genin–alkaline phosphatase-conjugated Fab fragments (CSPD; Boehringer Mannheim).

2.6. Protein preparations and ELISA

ELISAs were used to assess the presence of cytokine proteins in periapical tissues. For extract preparation, frozen samples were ground in a precooled sterile mortar and pestle, and the tissue fragments dissolved in 650–800 μl of lysis buffer consisting 100 μg/ml bovine serum albumin (fraction V; Sigma), 100 μg/ml Zwittergent-12 (Boehringer Mannheim), 50 μg/ml gentamycin (Life Technologies), 10 mM Hepes buffer (Life
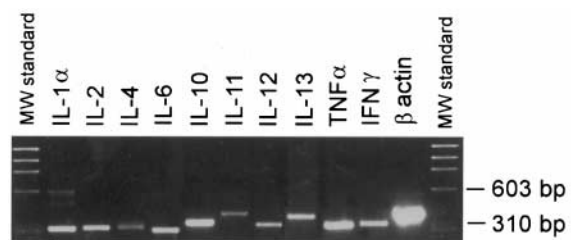

Fig. 1. RT-PCR amplification of cytokine mRNA from bovine serum albumin-immunized mouse spleen. RT-PCR products were electrophoresed on a 1.8% agarose gel stained with ethidium bromide. Lanes 1, 13, mol. wt marker, *Hae*III digested *Φ*X174 RF DNA; lane 2, IL-1α (290bp); lane 3, IL-2 (294bp); lane 4, IL-4 (293bp); lane 5, IL-6 (276bp); lane 6, IL-10 (324bp); lane 7, IL-11 (390bp); lane 8, IL-12 (310bp); lane 9, IL-13 (336bp); lane 10, TNFα (301bp); lane 11, IFNγ (310bp); lane 12, β-actin (382bp).

Technologies), 1 μg/ml aprotinin (Sigma), 1 μg/ml leupeptin (Sigma) and 0.1 μM EDTA (Fisher Scientific, Pittsburgh, PA) in RPMI 1640 (Mediatech, Herndon, VA), as described by Stashenko et al. (1991). The incubation mixture was placed on ice and sonicated for 20–30 sec. The supernatant was then collected and stored frozen at $-70°$C.

Assays for cytokines used commercially available ELISA kits according to the manufacturer's instructions: IL-1α (Endogen, Cambridge, MA; sensitivity 6 pg/ml); IL-2 (13 pg/ml), IL-4 (5 pg/ml), IL-6 (8 pg/ml), IL-10 (13 pg/ml), IL-12 (2 pg/ml), IFNγ (1 pg/ml) and TNFα (3 pg/ml) (all from BioSource International, Camarillo, CA). Immunized spleen served as a positive control. The concentration of each cytokine present in samples was calculated with reference to a standard curve that was constructed using recombinant cytokines provided with each kit. Results were expressed as pg cytokine/mg periapical tissue.

2.7. Statistical analysis

ELISA data were analysed by the non-paired Students' *t*-test with Bonferroni correction for multiple comparisons. To explore possible relations between cytokines, ELISA data were displayed in scatter plots and subjected to regression analysis.

3. Results

3.1. Expression of cytokine mRNA in periapical tissue

Qualitative RT-PCR was done on the total cellular RNA extracted from periapical lesions of each animal to assess the expression of cytokine mRNA. Positive-control amplification of all cytokines from immunized spleen revealed ethidium bromide-stained amplicons of the expected sizes (Fig. 1). All samples were positive for amplification of β-actin, demonstrating the integrity of the mRNA in the preparations, as well as the RT-PCR process (not shown). For periapical tissues, the expression of cytokine mRNA at each stage was expressed as the frequency of its detection (Table 2).

3.1.1. Bone resorptive cytokines

mRNA for IL-1α and TNFα were significantly upregulated after pulp exposure, IL-11 was constitutively expressed in periapical tissue or bone, and IL-6 mRNA was only occasionally detectable. IL-1α mRNA was expressed in all samples of periapical tissue after the exposure, but was also found in 5/11 control samples (Table 2). TNFα mRNA was also present in all samples after the exposure, but amplicons were absent from unexposed control periapical tissues (day 0). Approximately one-half of the samples (6/11)

showed expression of IL-11 mRNA in normal periapical tissue, a frequency which did not change significantly after pulp exposure. IL-6 mRNA was virtually undetectable, except for a single sample on each of days 14 and 28.

3.1.2. Th1-type cytokines

The expression of mRNAs for Th1 cytokines was upregulated by pulpal infection, and increased linearly over the period of observation. Little expression of IL-12 mRNA was observed in normal periapical tissues, whereas positive expression was seen on days 7, 14, and 28 (Table 2). Some expression of IFNγ mRNA was detected in normal tissues and the frequency of positive samples increased on days 14 and 28. No IL-2 mRNA was observed on day 0, but this cytokine was expressed on days 7 and 14 following pulp exposure.

3.1.3. Th2-type cytokines

Expression of Th2 cytokine mRNA was variably increased in periapical tissues in response to pulpal infection. IL-4 and IL-13 mRNA were expressed in some samples of normal (day 0) periapical tissue, but the frequency of expression remained nearly constant through the experimental period (Table 2). Expression of IL-10 mRNA was lower in normal periapical tissue, with only a modest elevation on day 14. As noted above, mRNA for IL-6, which also may function as a Th2 cytokine, was not highly expressed.

3.2. Detection of cytokine proteins in periapical tissues by ELISA

3.2.1. Bone-resorptive cytokines

Bone-resorptive cytokine proteins increased in response to pulpal infection, the clearest correlation

Table 2
Cytokine mRNA expression in the lesions

	Day 0	Day 7	Day 14	Day 28
IL-1α	5/11[a]	10/10	10/10	8/8
IL-2	0/7	3/10	4/10	1/9
IL-4	3/11	4/8	3/10	5/10
IL-6	0/7	0/9	1/10	1/9
IL-10	1/11	2/10	4/10	1/8
IL-11	6/11	7/10	6/10	7/9
IL-12	1/7	4/9	4/10	5/9
IL-13	4/11	3/10	3/9	2/9
IFNγ	4/11	3/10	6/10	7/9
TNFα	0/7	5/9	5/10	7/9

[a] Number of samples which showed the positive expression/total number of samples

being for IL-1α. As shown in Fig. 2, IL-1α protein was undetectable in normal periapical tissues despite the presence of mRNA (Table 2). However, periapical tissues on 7 and 14 days after pulpal exposure showed a statistically significant increase in IL-1α, with a further 1.5-fold increase on day 28. TNFα and IL-6 were both present in normal periapical tissues, and their expression further increased in the lesion after pulp ex-

Bone Resorptive Cytokines

Up-regulatory Cytokines

Down-regulatory Cytokines

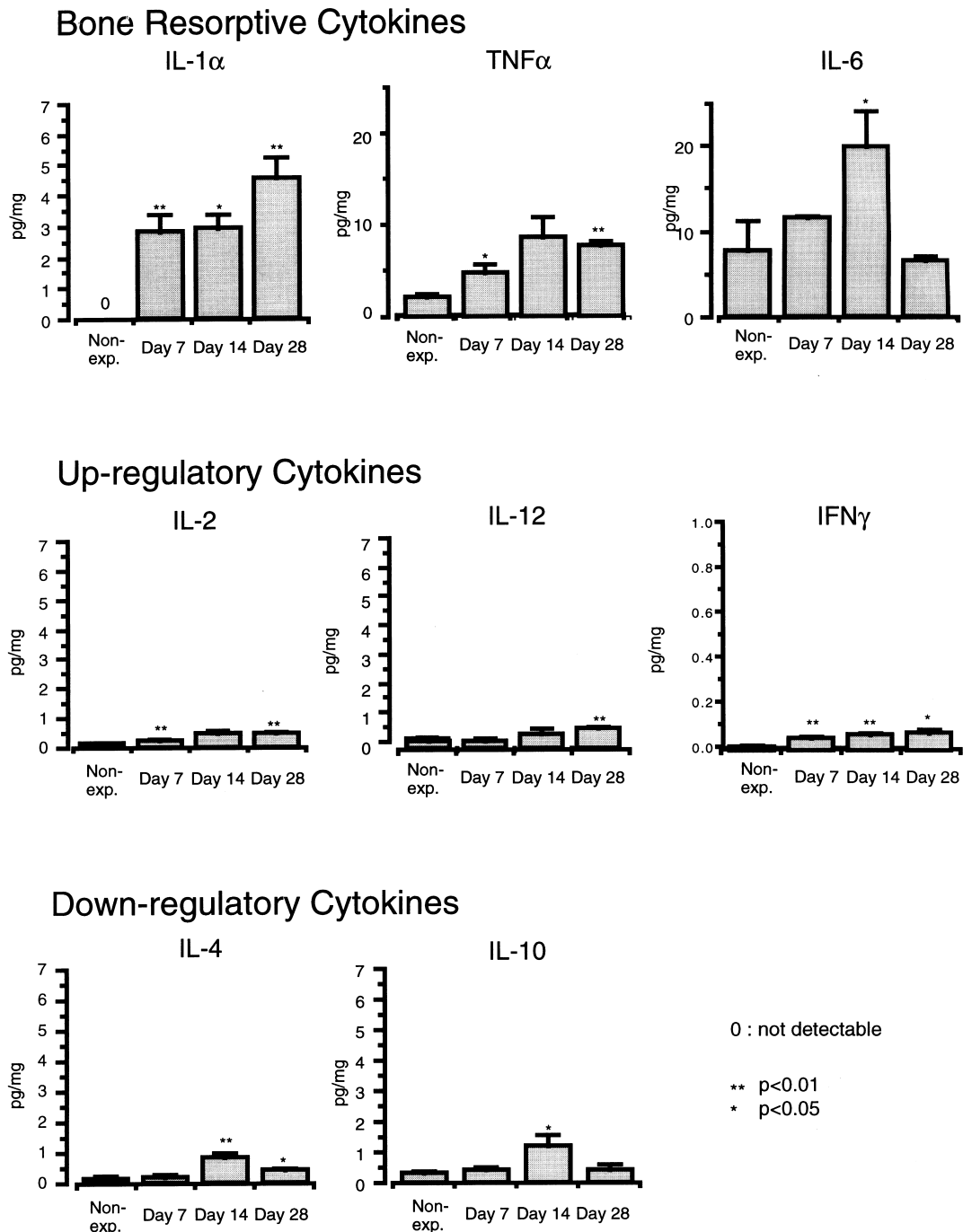0 : not detectable

** p<0.01
* p<0.05

Fig. 2. Quantitation of cytokines in periapical lesions by ELISA. Results are expressed as pg cytokine/mg periapical tissue. Horizontal bars indicate SEM. Differences from the non-exposed control were analysed by non-paired Student's' *t*-test with Bonferroni correction for multiple comparisons.

posure. The amounts of TNFα were significantly higher than in the normal samples on days 7 and 28. Despite the low levels of detectable mRNA, IL-6 protein was also induced by infection, with a significant increase seen on day 14. Of interest, a significant decline in IL-6 was observed on day 28, suggesting that the increase in this mediator is transient and may be downregulated (Fig. 3). IL-11 was not assessed as no assay is available.

3.2.2. Th1-type cytokines

The levels of Th1-type cytokines increased linearly over the 4-week period following pulp exposure (Fig. 2). IL-2 rose significantly on day 7 and 28 compared to normal tissue. IFNγ and IL-12 both showed a similar pattern of increase. For IFNγ, the increments from baseline were significant on days 7, 14, and 28, whereas for IL-12 they were significant on day 28. Of note, the overall concentrations of Th1-type cytokines were rather modest compared to those of the bone-resorptive mediators. In addition, the concentration of IFNγ was lowest of all the mediators assessed in this study.

3.2.3. Th2-type cytokines

IL-4 and IL-10 were present in low to moderate amounts in normal periapical tissues, but showed increases after pulp exposure (Fig. 2). For IL-4, these changes were significant on days 14 and 28. A nearly identical trend existed for IL-10, with a significant elevation on day 14 compared to control. Of interest, both mediators declined on day 28 compared to day 14, a similar pattern to that observed for the multifunctional mediator IL-6.

3.3. Correlation between expression of individual cytokines

A hypothesis tested in these studies is that the expression of Th1-type cytokines induces the expression of bone-resorptive mediators, particularly IL-1α, whereas Th2-type mediators are inhibitory. In order to explore these and other interrelations, correlations were sought between the cytokines quantified by ELISA, using scatter plots and regression analysis. Examples of such analyses are illustrated in Fig. 3 for the relationship between IL-1α and -12, and IL-4 and -10. The results of the regression analyses for all mediators are summarized in Table 3. As indicated, there were significant correlations between the amounts of IL-1α in periapical tissues, and Th1-type pro-inflammatory cytokines, particularly IL-12, IL-2, and to a borderline extent IFNγ. There was also a strong correlation between the amounts of TNFα and IL-1α, which are known to induce each other's production. Conversely, there was a lack of significant correlation

between IL-1α and Th2-type anti-inflammatory mediators, including IL-4, -6, and -10. However, there were strong associations amongst the Th1-type (IL-2, -12) and Th2-type (IL-4, -6, -10) cytokines themselves. Somewhat surprisingly, TNFα was positively related to the amounts of both Th2 and Th1 cytokines.

4. Discussion

In this investigation, we have determined the kinetics of expression of bone-resorptive and regulatory cytokines in a newly developed mouse model of the periapical lesion. We show a clear relationship between the expression of IL-1α mRNA and protein and periapical bone destruction, in agreement with our previous findings in the rat (Wang and Stashenko, 1993; Tani-Ishii et al., 1995; Wang et al., 1997). Bacterial infection of the pulp stimulates resident macrophages and fibroblasts, and induces the infiltration of macrophage/monocytes (Okiji et al., 1994; Kawashima et al., 1996) which secrete IL-1α (Stashenko et al., 1994; Tani-Ishii et al., 1995). A relatively large amount of IL-1α production was already observed on day 7 after pulp exposure, which is the earliest stage of periapical bone resorption in this model. We did not evaluate IL-1β, primarily because its expression in rodents is much lower than that of IL-1α, including in infected pulp and periapical tissues (Tani-Ishii et al., 1995; Wang et al., 1997). Some IL-1α mRNA was seen in normal periapical tissues, but protein was not detected, suggesting a lack of mRNA translation. There is some expression of IL-1 in normal tissues by fibroblasts and resident macrophages (Hsu and Zhao, 1987; Mauviel et al., 1988). Taken together with previous findings (Stashenko et al., 1994), these results indicate that IL-1α plays a crucial part in bone destruction and lesional expansion, although the bioactivity of IL-1α may be modified by IL-1receptor antagonist and IL-1 receptor type II, which were not assessed here (Dinarello, 1994).

TNFα mRNA and protein also clearly increased in response to pulpal infection, similar to previous findings in the rat (Wang et al., 1997). The high correlation between TNFα and IL-1α is probably related to their co-stimulatory effects (Kaushansky et al., 1988). The concentration of TNFα in lesional tissues was somewhat greater than that of IL-1α. However, neutralization studies in the rat have indicated that TNFα does not contribute significantly to periapical bone resorption (Wang and Stashenko, 1993), probably because its potency is 25-fold lower in stimulating osteoclasts than that of IL-1α (Stashenko et al., 1987). Nonetheless, TNFα may promote bone destruction indirectly through the induction of IL-1α. TNFα protein was present in normal periapical tissue, although
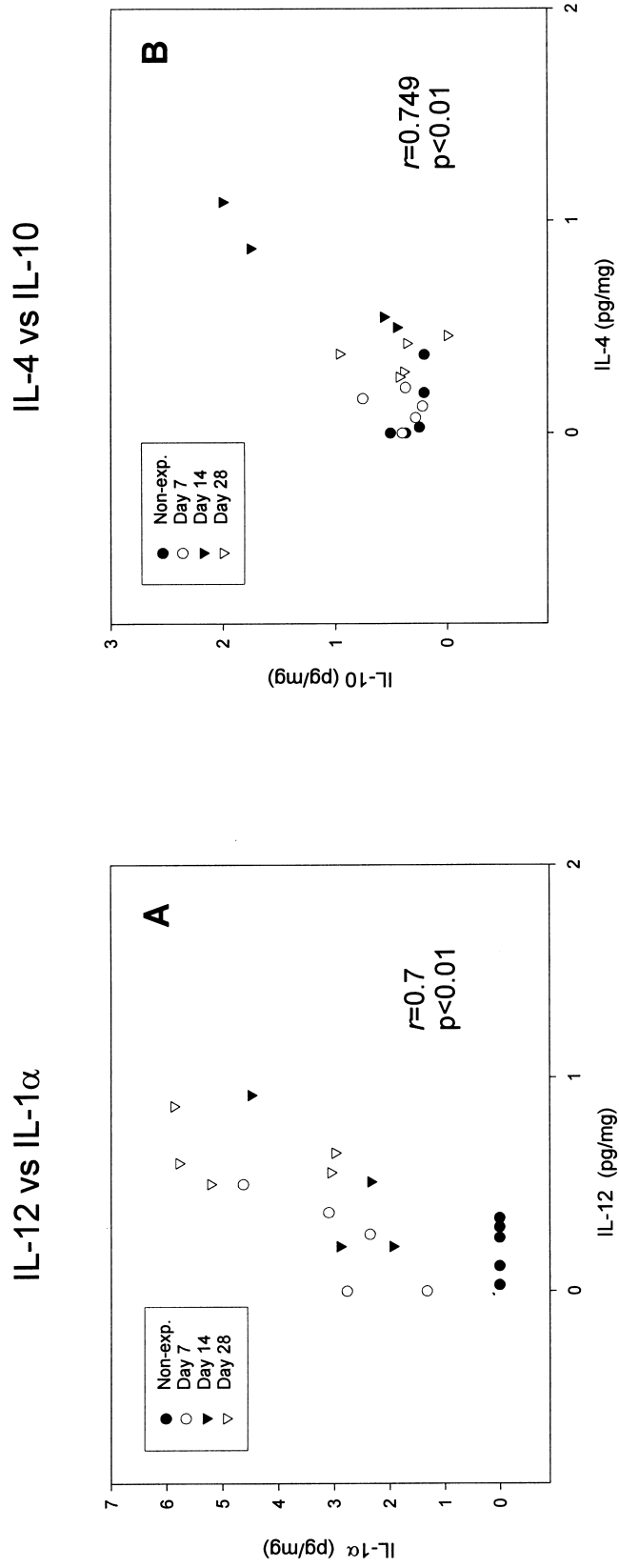
IL-4 vs IL-10

IL-12 vs IL-1α

Fig. 3. Correlations between expression of individual cytokines. The cytokine contents (pg/mg) of individual periapical tissue samples as determined by ELISA were plotted and the significance of correlations determined by regression analysis. (A) IL-12 vs IL-1α; (B) IL-4 vs IL-10.

Table 3
Correlation between expression of cytokines

	IL-1α	IL-2	IL-4	IL-6	IL-10	IL-12	IFNγ
IL-2	**0.631103**[a,b]						
IL-4	0.3750228	**0.6562353**					
IL-6	0.1073815	*0.539815*[c]	**0.677478**				
IL-10	0.2736509	**0.7019482**	0.748707	0.877595			
IL-12	**0.7000406**	0.6373665	*0.487532*	0.278086	0.393334		
IFNγ	*0.4759129*	0.5099082	0.341836	0.176069	0.168005	0.389888	
TNFα	**0.6882025**	*0.5728025*	**0.667152**	*0.460746*	**0.608129**	0.583962	0.402734

[a] The number shows the value of *r*.
[b] **bold**: cytokines significantly correlated; $P < 0.01$.
[c] *italic*: cytokines significantly correlated; $P < 0.05$

mRNA was not detected. A possible explanation may be that adjacent bone cells constitutively produce TNFα which is involved in normal bone homeostasis, and that this mediator subsequently diffuses into the lesion (Gowen et al., 1990; Marie et al., 1993; Modrowski et al., 1995).

IL-6 and IL-11 are related through their sequence homology, chromosomal location, and receptors (Romas et al., 1996), and exert their functions through a common, 130-kDa, signal-transducing subunit (gp 130) (Taga et al., 1989; Zhang et al., 1994; Romas et al., 1996). We observed the production of IL-6 in normal periapical tissue and an increase after pulp exposure, although we mostly failed to detect IL-6 mRNA. It is conceivable that, similar to IL-4 (Yamamoto et al., 1997), the mRNA for IL-6 may have an extremely short half-life and may therefore be difficult to amplify by PCR. Alternatively, IL-6 protein may not have been produced within the periapical lesion, but may have localized there from the circulation, as suggested by Vargas et al. (1996), who detected IL-6 protein in the bone marrow in the absence of IL-6 mRNA. Further studies are needed to clarify this issue. Some studies have shown that IL-6 has direct stimulatory effects on bone resorption (Ishimi et al., 1990), although this is controversial (Lowik et al., 1989; al-Humidan et al., 1991). IL-1 and TNF stimulate the production of IL-6 and (IL-11) (Elias and Lentz, 1990; Girasole et al., 1992). On the other hand, IL-6 stimulates the production of IL-1 receptor antagonist (Tilg et al., 1994), which inhibits the actions of IL-1 toward its target cells, and furthermore may act to inhibit IFNγ as a down regulatory, Th2-type mediator. Several groups report the presence of IL-6 in human periapical lesions and cysts (Bando et al., 1993; Formigli et al., 1995; Meghji et al., 1996), and the amounts of IL-6 have been correlated with the extent of tissue damage and bone destruction in periodontitis (Prabhu et al., 1996). IL-6 is a multifunctional cytokine which may be involved in both osseous homeostasis and inflammation.

To the best of our belief, IL-11 has not previously been reported in periapical or periodontal tissues. IL-11 mRNA was found in half of the normal samples, a frequency that did not significantly increase with time after pulp exposure. This finding indicates that inflammation had little stimulatory effect on IL-11. Rather, the presence of this mediator in normal periapical tissue suggests that IL-11, which is produced from osteoblasts and stimulates the proliferation and differentiation of osteoclast precursors, may be essential for normal bone remodelling (Elias et al., 1995; Romas et al., 1996). However, Trepicchio et al. (1996) showed that IL-11 has an inhibitory effect on the production of pro-inflammatory cytokines, so that its overall effects in an inflammatory site are, like that of IL-6, difficult to predict.

Expression of IL-2 was low in periapical lesions, but increased with time after pulp exposure. We previously showed that T-lymphocyte infiltration in rat periapical lesions begins at approx. 7–14 days after pulp exposure (Kawashima et al., 1996), and the increase in IL-2 is probably derived from these cells. IL-2 is mainly produced by CD4+ Th1 cells, and induces growth, activation and cytokine production of T cells. These activities are illustrated by the high correlation between IL-2 and IL-4, and IL-10 (Table 3). IL-2 is generally considered to lack direct effects on bone resorption (Gowen and Mundy, 1986).

The frequency of IL-12 and IFNγ mRNA and levels of protein expression were also increased with time after pulp exposure. IL-12, which is mostly produced by macrophages as well as Th1 cells, induces the functional development of Th1 cells from naïve T cells and activates Th1 cells to produce IFNγ. Whereas IL-12 has not been reported to have direct effects on osteoclasts, IFNγ has apparently contradictory effects on resorptive activity. It activates macrophages to produce increased amounts of resorptive cytokines such as IL-1

and TNFα (Arenzana-Seisdedos et al., 1985; Collart et al., 1986), but also inhibits osteoclast formation, development and function in vitro (Lacey et al., 1995; Takahashi et al., 1986). Gowen et al. (1986) showed that IFNγ inhibits resorption induced by IL-1 and TNFα on mouse calvaria. Recently, it was reported that IFNγ selectively inhibits cytokine-induced bone resorption by generating large amounts of NO, which exerts a biphasic regulation of osteoclast-mediated bone resorption with stimulation at low concentrations and inhibition at high concentrations (Ralston et al., 1995). On the other hand, IFNγ in vivo causes the formation of osteoclasts and their synthesis of superoxides (Key et al., 1992, 1995; Klaushofer et al., 1989). In rats, intraperitoneal injection of IFNγ induced osteopenia (Mann et al., 1994). On balance therefore, it appears that the net effect of IFNγ in vivo is to stimulate resorption, although more direct methods of analysis will be required to resolve this point.

The Th2-type cytokines IL-4, -10 and -13 generally possess inhibitory properties on bone resorption. They modulate the biosynthesis of pro-inflammatory cytokines and regulate the production of their antagonists. Th2 cells are the major source of IL-4 (Mossman and Coffman, 1989), although mast cells and other cell types may also produce this mediator (Horsmanheimo et al., 1994). IL-4 reportedly inhibits osteoclast formation (Riancho et al., 1993; Lacey et al., 1995) and bone resorption (Bizarri et al., 1994; Miossec et al., 1994). Onoe et al. (1996) found that both IL-4 and IL-13 inhibit resorption by suppressing the expression of cyclo-oxgenase-2 mRNA and the production of prostaglandins in osteoblasts. IL-10, which is produced by macrophages (Fiorentino et al., 1991), Th0 cells (Windhagen et al., 1996) and Th2 cells (Fiorentino et al., 1989), inhibits cytokine production from activated T cells and macrophages, and induces Th2-type immune responses. IL-10 reportedly inhibits osteoclast formation (Owens et al., 1996). In the present study, IL-4 and IL-10 proteins were significantly increased on [days 7 and 14]. In vitro, IL-4 inhibited osteoclast-like cell formation induced by IL-1α on an equimolar basis, indicating that the concentrations of the inhibitory cytokines may be sufficient to exert an effect on inflammatory resorption (Kasono et al., 1993). Of interest, there was a trend for the amounts of IL-4 and -10 as well as IL-6 to decline on day 28 compared to day 14, whereas the upregulatory cytokines IL-12 and IFNγ continued to rise. This observation suggests that the anti-inflammatory cytokines are induced to similar levels as upregulatory cytokines in response to pulpal infection, but that expression is transient and may even be counter-regulated by the anti-inflammatory cytokines as the lesion progresses.

Correlations among the cytokines examined lend support to the hypothesis that a cytokine network, some of the elements of which have been defined in this study, is operative in regulating infection-stimulated bone resorption. There were strong correlations between IL-1α and IL-2, IL-12, and a borderline effect with IFNγ, whereas there was no positive relations with IL-4, -6, or -10. There was a lack of correlation between Th1-type (IL-12, IFNγ) and Th2-type (IL-6, IL-10) cytokines. Based upon these findings, we propose that inflammatory bone resorption may be upregulated in vivo by Th1-type mediators, and downregulated by Th2-type mediators. Given the central role of IL-1 in the pathogenesis of not only periapical lesions but also other bone-destructive diseases, including rheumatoid arthritis, osteoporosis and periodontitis, methods for controlling IL-1 function directly and/or indirectly may provide a means of treating these diverse disorders.

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