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Interleukin-4 Suppresses Interleukin-1-Induced Expression of Matrix Metalloproteinase-3 in Human Gingival Fibroblasts

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Abstract

OBJECTIVE—In periodontitis, matrix metalloproteinase 3 (MMP-3, stromelysin 1) is present at increased levels in active disease sites compared to inactive or healthy sites, and the levels are correlated with clinical parameters and associated with progression of the disease. Interleukin 4 (IL-4) has been shown in other systems to suppress interleukin-1 (IL-1) induced expression of MMP-3, but this has not been shown in human gingival fibroblasts. The objective of this study is to determine the effects of IL-4 on the IL-1 induced expression of MMP-3 in human gingival fibroblasts isolated from patients with periodontitis.

METHODS—Northern blot analysis was performed to determine the effects of IL-4 on the IL-1 induction of MMP-3 mRNA. MMP-3 protein levels were determined by ELISA, and prostaglandin E_2 (PGE2) levels were measured by enzyme immunoassay (EIA). DNA binding of AP-1 and NF-κB was assessed by electrophoretic mobility shift assay (EMSA).

RESULTS—Northern blot analysis revealed that co-incubation of gingival fibroblasts with IL-1 and IL-4 resulted in a significant decrease in MMP-3 mRNA levels compared to IL-1 alone, with a concomitant decrease in protein levels. This inhibition is dose dependent, and is apparent as early as 3 hours after stimulation. IL-1-induced production of PGE2 was not affected in 4 of 6 cultures isolated from different individuals. Addition of exogenous PGE2 had no effect on the suppressive effects of IL-4. DNA binding of transcription factors AP-1 and NF-κB was not affected by IL-4.

CONCLUSION—IL-4 inhibits the IL-1 induction of MMP-3 in human gingival fibroblasts isolated from patients with periodontitis. This effect is independent of PGE2 and is not due to inhibition of the DNA binding activity of known transcription factors binding to the MMP-3 promoter.

Keywords

Periodontitis; Stromelysin-1; Gene expression regulation; Interleukin-4; Interleukin-1

INTRODUCTION

Periodontitis is the most common cause of adult tooth loss in the U.S. [1], with an estimated 1 in 3 adults suffering from some form [2]. In addition to its direct impact, periodontitis may also contribute to the development of several other diseases, including cardiovascular disease, pre-term low birth weight and diabetes [3,4]. Bacteria are essential for initiation of periodontitis, but host factors are largely responsible for the development of a chronic inflammatory state leading to destruction of periodontal support structures [5].

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Chronic inflammation in periodontitis is characterized by increased levels of IL-1β, TNFα and prostaglandin E2 (PGE2) [6–8]. Interestingly, however, there seems to be a local T cell imbalance, with a relative absence of IL-4 producing T cells at sites of periodontal inflammation [9–11]. This imbalance appears to be progressive, with decreasing levels of IL-4 correlated with loss of collagen and with increasing clinical severity [12]. In addition, polymorphisms in the IL-4 promoter and intron that are associated with decreased serum levels of IL-4 are also associated with increased susceptibility to early onset periodontitis [13]. It has been suggested that correcting this cytokine imbalance in chronic inflammatory conditions might be therapeutic. In fact, adenoviral transfer of IL-4 has been shown to be protective against cartilage degradation induced by injection of rheumatoid arthritis synovial tissue into joints of SCID mice [14] and against collagen-induced arthritis [15]. However, a better understanding of the mechanisms of IL-4's beneficial effects might make possible safer and more economical therapies.

MMP-3 (Stromelysin-1) is a metalloproteinase with broad substrate specificity, degrading proteoglycan, laminin, fibronectin, and the non-fibrillar collagens [16]. Perhaps equally important, it is also capable of activating other pro-MMPs, including MMP-1, -8, -9 and –13 [17–21], of inactivating plasminogen activator inhibitor I [22] and of cleaving FasL to produce sFasL [23]. MMP-3 is produced by gingival and synovial fibroblasts, chondrocytes, macrophages, neutrophils, and endothelial cells in response to inflammatory cytokines and mitogens. In periodontitis, MMP-3 is present at increased levels in active disease sites compared to inactive or healthy sites [24–29], and the levels are correlated with clinical parameters and associated with progression of the disease [28].

IL-4 has been shown to inhibit the IL-1 induction of MMP-3 expression in human skin fibroblasts [30] and articular chondrocytes [31,32], as well as in human synovial fibroblasts [33]. However, effects of IL-4 on MMP-3 expression have not been demonstrated in cells relevant to periodontitis. Here, we show that IL-4 also inhibits the IL-1-induced expression of MMP-3 mRNA and protein in human gingival fibroblasts (HGF) isolated from patients with periodontitis. This effect appears to be independent of any effects on production of PGE2 or DNA binding of transcription factors known to regulate MMP-3 expression.

MATERIALS AND METHODS

Cell culture

Human gingival tissue from patients undergoing periodontal surgery was obtained from Howard M. Sobel, D.D.S. and Kevan S. Green, D.M.D. of Sobel Periodontal Associates, P.C. The tissue was processed by enzymatic dispersion to produce primary cultures [34,35]. Cells were maintained in Eagle's Minimal Essential Medium (EMEM) supplemented with 10% fetal bovine serum and antibiotic/antimycotic (penicillin, streptomycin, amphotericin; Gibco BRL, Grand Island, NY). Cells between passages 3 and 5 were used for experiments. Cells were serum-deprived for 16 hours in serum-free EMEM supplemented with 10% ITS (insulin, transferrin, sodium selenite; Sigma, St. Louis, MO) prior to the addition of 100 ng IL-1β/ml (a gift of R. Newton, Wilmington, DE) in the presence or absence of various doses of IL-4 (Gibco BRL, Grand Island, NY).

RNA Isolation and Northern Blotting

Total RNA was isolated according to the acid-phenol method of Chomczynski and Sacchi [36] at various times after treatment, and run on 1% agarose-formaldehyde gels. Probes were made by random priming (Stratagene, La Jolla, CA) of cDNA fragments corresponding to rat stromelysin-1 (MMP-3, American Type Culture Collection, Rockville, MD) and

glyceraldehyde 3 phosphate dehydrogenase (GAPDH, a gift of R. Newton, Wilmington, DE). Northern blots were quantitated by densitometric scanning and normalized to GAPDH.

Quantitation of MMP-3 Protein and Prostaglandin E2

MMP-3 protein levels were quantitated by ELISA (Amersham, Arlington Heights, IL) in conditioned media of human gingival fibroblasts (HGF) that were untreated, treated with 100 ng IL-1/ml alone or treated with both 100 ng IL-1/ml and 10 ng IL-4/ml for 24 hours. Levels of PGE2 were measured by EIA (Amersham) in 6 hour conditioned media.

Nuclear Extract Isolation and EMSA

Nuclear extracts were isolated according to the method of Schreiber et al. [37] and quantitated in mini-Bradford assays (Pierce, Rockford, IL). Synthetic oligonucleotides corresponding to the consensus binding sites for NF-κB and AP-1 (Santa Cruz, Biotechnology, Inc., Santa Cruz, CA) were labeled by T4 polynucleotide kinase in the presence of γ^{32}P-ATP. Binding reactions contained 5 μg protein, 20 mM Hepes-OH pH 7, 50 mM NaCl, 0.2 M EDTA, 5% glycerol, 4 μg dIdC and 10,000 cpm probe.

RESULTS

IL-4 inhibits the IL-1 induction of MMP-3 mRNA and protein

In order to determine the effect of IL-4 on the IL-1 induction of MMP-3 mRNA expression in human gingival fibroblasts (HGF), total RNA was isolated at various times after addition of either IL-1 alone or IL-1 simultaneously with IL-4. Northern blot analysis revealed a significant reduction in the IL-1 induced expression of MMP-3 mRNA, approximately 70% at 6 hours (Figure 1). This inhibition was evident as early as 3 hours after stimulation.

Incremental doses of IL-4 were added simultaneously with a constant dose of IL-1 for dose curve analysis. Total RNA was isolated 6 hours after stimulation, and Northern blot analysis was performed to determine the effects of each dose on the IL-1 induction of MMP-3. Figure 2 shows that the suppressive action of IL-4 is dose dependent, with some inhibition seen with 0.1 ng IL-4/ml, and maximal inhibition was seen with 50 ng IL-4/ml. The IC_{50}, as determined by regression analysis of this data, was approximately 0.3 ng/ml. These results are very similar to those seen in synovial fibroblasts [33].

Levels of MMP-3 protein were measured in conditioned media from 6 different HGF cultures derived from different donors, which were treated with IL-1 alone or co-incubated with 10 ng/ml IL-4 for 24 hours. There was variation among the 6 cultures in both basal and IL-1 induced levels of MMP-3. The extent of IL-4 suppression of the IL-1 induction of MMP-3 protein varied as well, from essentially no inhibition to over 90% (Figure 3). Taken together, however, there was an average inhibition of ~ 54% ($p<0.01$), and this rises to 65% ($p < 0.01$) if culture #2 is excluded as an outlier.

IL-4 inhibition of IL-1 induced MMP-3 production is not always associated with decreased production of prostaglandin E2

IL-4 has been reported to inhibit IL-1 induced production of PGE2 in several model systems [33,38–41], including human gingival fibroblasts and periodontal ligament fibroblasts isolated from individuals with healthy periodontia [42], and in some cases the inhibition of PGE2 production has been linked to IL-4's suppressive effects on gene expression. However, there are conflicting reports on the role of PGE2 in IL-1 induced production of MMP-3 [43–50]. In order to determine whether IL-4 inhibition of MMP-3 production is associated with changes in PGE2 levels in HGF, levels of PGE2 were measured in 6 hour conditioned media from 6 different HGF cultures (derived from 6 different donors), stimulated with IL-1 in the presence

or absence of IL-4. Interestingly, IL-4 inhibited the IL-1 induced production of PGE2 in only 2 of the 6 HGF cultures (Figure 4), and on average, there was no effect. Furthermore, addition of exogenous PGE2 along with IL-1 and IL-4 had no effect on the ability of IL-4 to inhibit the IL-1 induction of MMP-3 (Figure 5).

Binding of transcription factors AP-1 and NF-κB is not affected by IL-4

Transcription factor AP-1 is an important factor in the regulation of the MMP-3 gene [51–54] and there is evidence that NF-κB may also play a role [55–57]. Since IL-4 has been shown in other systems to inhibit gene expression by interfering with AP-1 [58,59] and NF-κB activation [60–62], the effects of IL-4 on AP-1 and NF-κB DNA binding were investigated. Nuclear extracts were isolated from HGF cultures one hour after addition of IL-1 alone, IL-4 alone, or IL-1 in the presence of IL-4. As shown in Figure 6, IL-4 had no effect on AP-1 or NF-κB DNA binding activity. In addition, binding to other promoter elements, the polyoma virus enhancer 3 (PEA-3) site [63] and the stromelysin IL-1 responsive element (SIRE) [64], was also unaffected by IL-4 (data not shown).

DISCUSSION

Chronic inflammatory conditions such as periodontitis and rheumatoid arthritis result in tissue destruction due in large part to local over-expression of inflammatory mediators, including MMPs and prostaglandins. Here we show that the anti-inflammatory cytokine IL-4 has a dose-dependent inhibitory effect on the IL-1 induction of MMP-3. Although similar results have been reported in human skin and synovial fibroblasts [30,33,65] and articular chondrocytes [31, 32], this to our knowledge is the first report of such findings in cells relevant to periodontitis.

Although the present data do not address the issue of whether or not the inhibition takes place at the transcriptional level, they are consistent with that conclusion. MMP-3 expression is regulated primarily at the transcriptional level, and previous results showed that IL-4 inhibited IL-1 induced transcription from the MMP-3 promoter in transiently transfected human foreskin fibroblasts [33]. However, IL-4 has also been shown to suppress gene expression by decreasing mRNA stability [66], and that possibility cannot be excluded.

Several previous reports showing IL-4 inhibition of gene expression have focused on inhibition of prostaglandin synthesis, presumably leading to decreased production of cAMP [40,41,67, 68]. However, Sugiyama et al. [41] found that IL-4 inhibition of IL-1α induced cyclooxygenase II mRNA and PGE2 production was cell-type specific, occurring in PMA-differentiated U937 cells and freshly prepared adherent synoviocytes, but not in rheumatoid synovial fibroblasts. In addition, there are several conflicting reports concerning the role of prostaglandins in the IL-1 induction of MMP-3. Inhibition of prostaglandin synthesis by indomethacin or other inhibitors of cyclooxygenase has been shown to both augment [50] and inhibit [47,69] MMP-3 expression, as has exogenous addition of PGE2 [46,48–50] and alterations in levels of cAMP [48–50].

Hayashi et al. [42] found that IL-4 inhibits IL-1 induced production of PGE2 in three different types of normal fibroblasts, including periodontal ligament and gingival fibroblasts. Our results, in contrast, show failure of IL-4 to inhibit PGE2 production by 4 of 6 gingival fibroblast cultures (Figure 4). There is currently no clear explanation of these results. However, it must be reiterated that our HGF cultures were derived from patients with periodontitis, whereas those of Hayashi et al. were derived from tissue from healthy periodontia. Although simple variation among individuals cannot be excluded based on these small sample sizes, it is possible that cells isolated from chronically inflamed tissue have been altered in a way that interferes with normal responses to IL-4. One example of cells from diseased tissue exhibiting altered responses comes from the work of Millward-Sadler et al. [70]. They found that mechanical

stimulation of normal chondrocytes results in decreased expression of MMP-3 via an integrin-mediated, IL-4 dependent mechanism, but this was not the case in chondrocytes isolated from donors with osteoarthritis. Further studies suggest that IL-4 signaling in OA chondrocytes is preferentially through the type I (IL4α/cγ) receptor rather than via the type II (IL4α/IL13R) receptor [71]. The make-up of the IL-4 receptor on gingival fibroblasts, and whether or not it is altered in periodontitis is not known. It is clear however, that at least some aspects of IL-4 signaling are still intact, since IL-4 is able to inhibit MMP-3 expression in most, if not all, cultures.

It is also possible that some of the individual variation observed might be due to characteristics of the tissue donors. Our tissue samples were supplied without any information about the donors. However, both smoking and diabetes are strong risk factors for periodontitis [72], and could conceivably have effects on gingival cell properties and their response to cytokines. For example, exposure to volatile components of cigarette smoke alters the cytoskeleton and reduces cell adhesions of HGF [73], and nicotine interferes with the normal localization of β1 integrin to the plasma membrane [74]. Both smoking and diabetes can increase oxidant stress [75,76], which can activate transcription factors such as AP-1 and NF-κB [60–62], and treatment of human skin fibroblasts with tobacco smoke extract increased MMP-3 mRNA expression [77].

Our results further show that addition of exogenous PGE2 has no effect on IL-4's ability to suppress the IL-1 induction of MMP-3. These results are consistent with our earlier results with human synovial fibroblasts [33] and those of Prontera et al. [30], who showed that the IL-4 inhibition of IL-1 induced MMP-3 expression in human skin fibroblasts is independent of protein kinase A or cAMP levels. Taken together, these data suggest that even when IL-4 can inhibit production of PGE2, this is not causally related to its ability to inhibit expression of MMP-3.

The mechanisms involved in IL-4 suppression of MMP-3 expression are not known. Transcription factor AP-1 plays an important role in regulating transcription from the MMP-3 promoter in response to a variety of cytokines and mitogens, including IL-1 [51–54], as do members of the Ets family of transcription factors [63]. There is also some evidence that NF-κB may play a role [55–57]. IL-4 has been shown to affect gene expression by interfering with the DNA binding activity of AP-1 [58,59] and NF-κB [60–62] in other systems; however our results suggest that this is not the case in HGF. Interestingly, AP-1 binding activity seemed to be constitutively activated in these HGF. Similar results have been found in rheumatoid arthritis synovial fibroblasts [78,79]. IL-4 had no effect on AP-1 binding, either basal or in the presence of IL-1. NF-κB had a lower basal level of DNA binding, but IL-4 had no effect on basal or IL-1 induced binding. IL-1 induced binding of nuclear proteins to PEA3/Ets and stromelysin IL-1 responsive element (SIRE) binding sites was also not affected by IL-4 (data not shown).

The effects of IL-4 on gene expression are generally mediated through activation of STAT6, a member of the "signal transducers and activators of transcription" family [80]. STAT6 has been shown to exist in human synovial fibroblasts, and to be capable of activation by IL-4 [81], however it has not been studied in HGF. STAT6 induces transcription through interactions with the co-activator p300/CBP [82]. AP-1, NF-κB and Ets 1 and 2, as well as other transcription factors, also require p300/CBP, and their activity can be inhibited by factors such as STATs that compete for the co-activator [83–89]. Thus, IL-4 could be inhibiting transactivation of MMP-3 via a transcription factor without necessarily affecting its DNA binding activity. It is also possible that the inhibitory effects of IL-4 are mediated through other transcription factors or elements that have not yet been identified or characterized.

In summary, we have presented evidence that co-incubation of HGF with IL-4 and IL-1 results in dose-dependent reduction in the IL-1 induced production of MMP-3. Surprisingly, this inhibition was not consistently associated with decreased production of PGE2, and did not involve inhibition of DNA binding activity of transcription factors known to be involved in regulation of MMP-3 regulation. Further study is needed to address the mechanism of this inhibition and to address issues of individual variation in the cell cultures.

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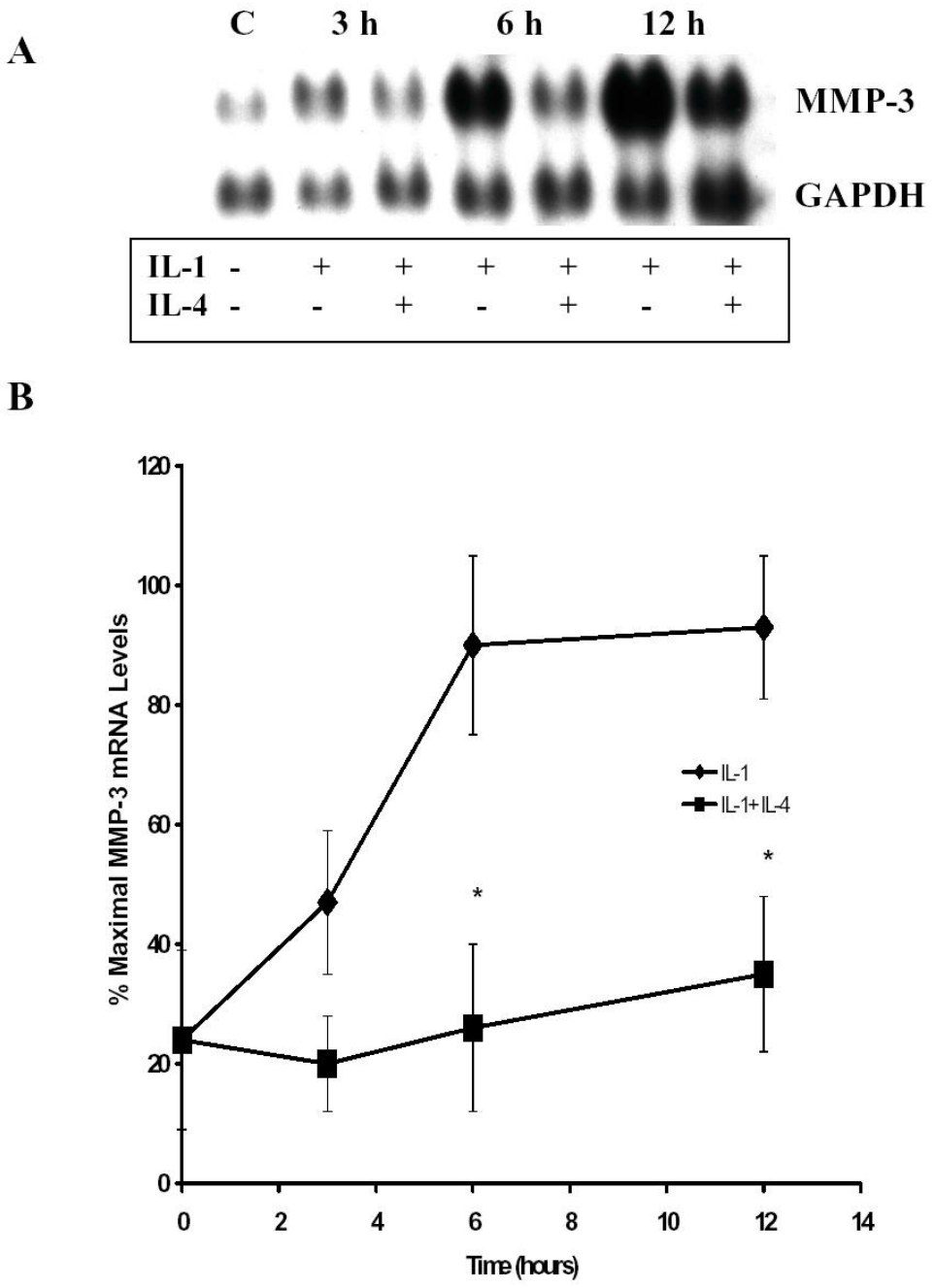

Figure 1.

Inerleukin-4 (IL-4) inhibits IL-1 induction of MMP-3 mRNA in human gingival fibroblasts (HGF). Total RNA was isolated from untreated HGF and cells treated for the indicated times with IL-1β alone (100 ng/ml) or in combination with 10 ng/ml IL-4. **A**. Northern blots were hybridized to cDNA probes corresponding to MMP-3 and GAPDH. **B.** Blots were quantitated by scanning densitometry and normalized to levels of GAPDH. Shown are data from three independent experiments, each of which utilized RNA isolated from three pooled HGF cultures established from three different donors. (* $p < 0.05$ vs. IL-1 alone).

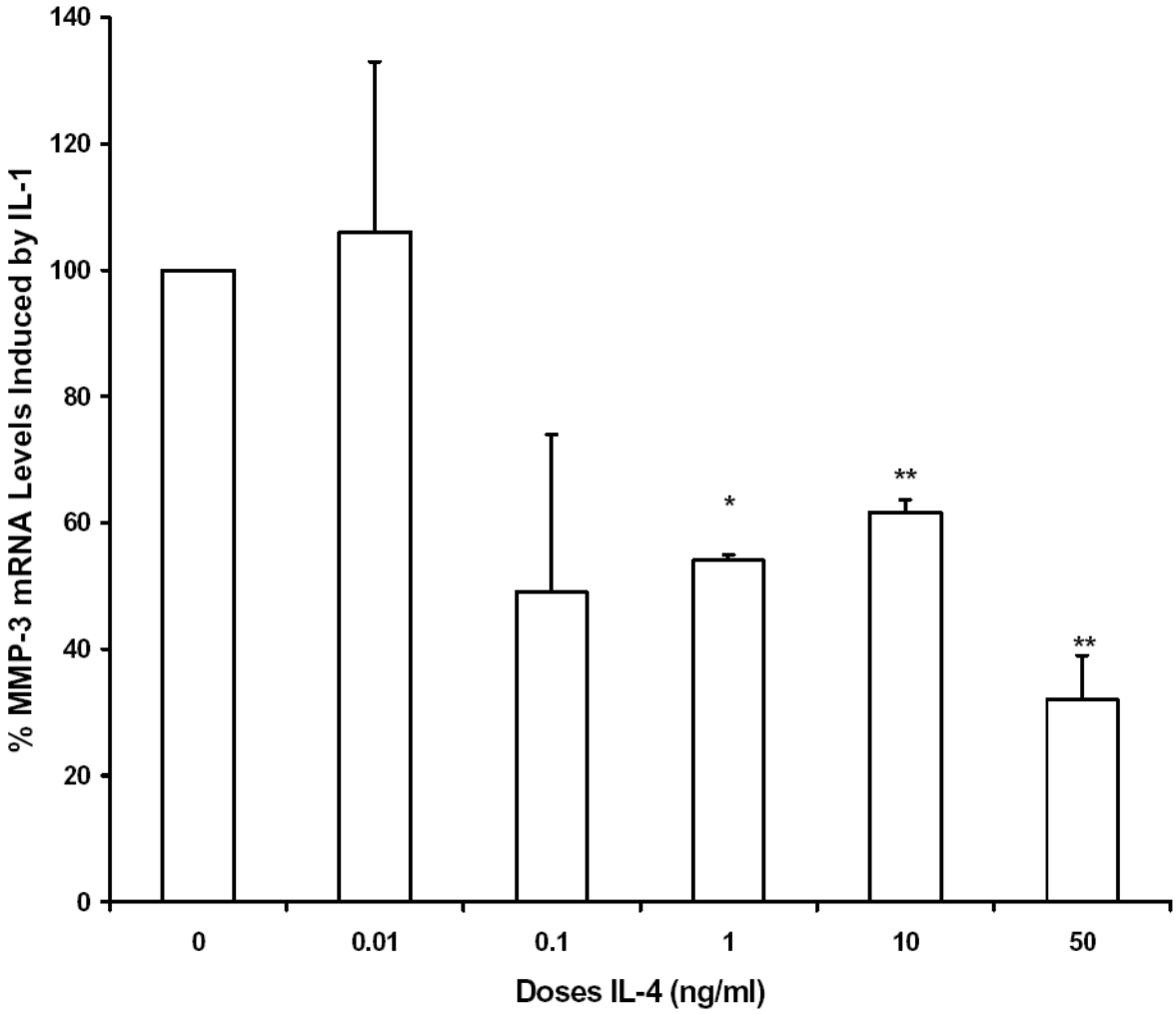

Figure 2.

Interleukin-4 (IL-4) inhibition of IL-1-induced expression of MMP-3 mRNA is dose dependent. Total RNA was isolated from HGF cultures 6 hours after addition of 100 ng/ml IL-1β alone or together with the indicated doses of IL-4. Shown are data from three independent experiments, each of which utilized RNA isolated from three pooled HGF cultures established from three different donors. (* $p < 0.05$; ** $p<0.01$ vs. IL-1 alone)

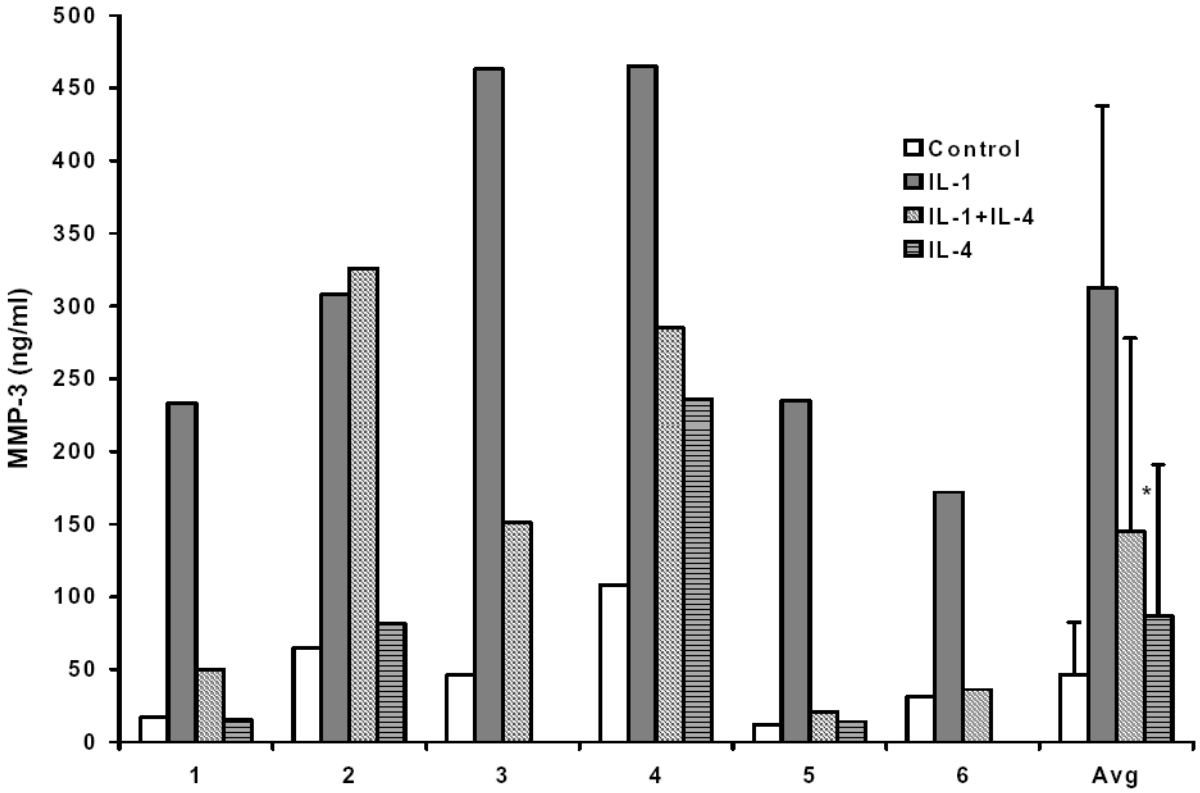

Figure 3.

Interleukin-4 (IL-4) inhibits the IL-1 induction of MMP-3 protein. Conditioned medium was harvested from HGF cultures incubated for 24 hours with 100 ng/ml IL-1β alone or in the presence of 10 ng/ml IL-4. Levels of MMP-3 protein were measured in triplicate by enzyme-linked immunosorbent assay. HGF cultures derived from six different individuals (numbered 1 through 6 on the graph) were used. (* $p < 0.05$ vs. IL-1 alone).

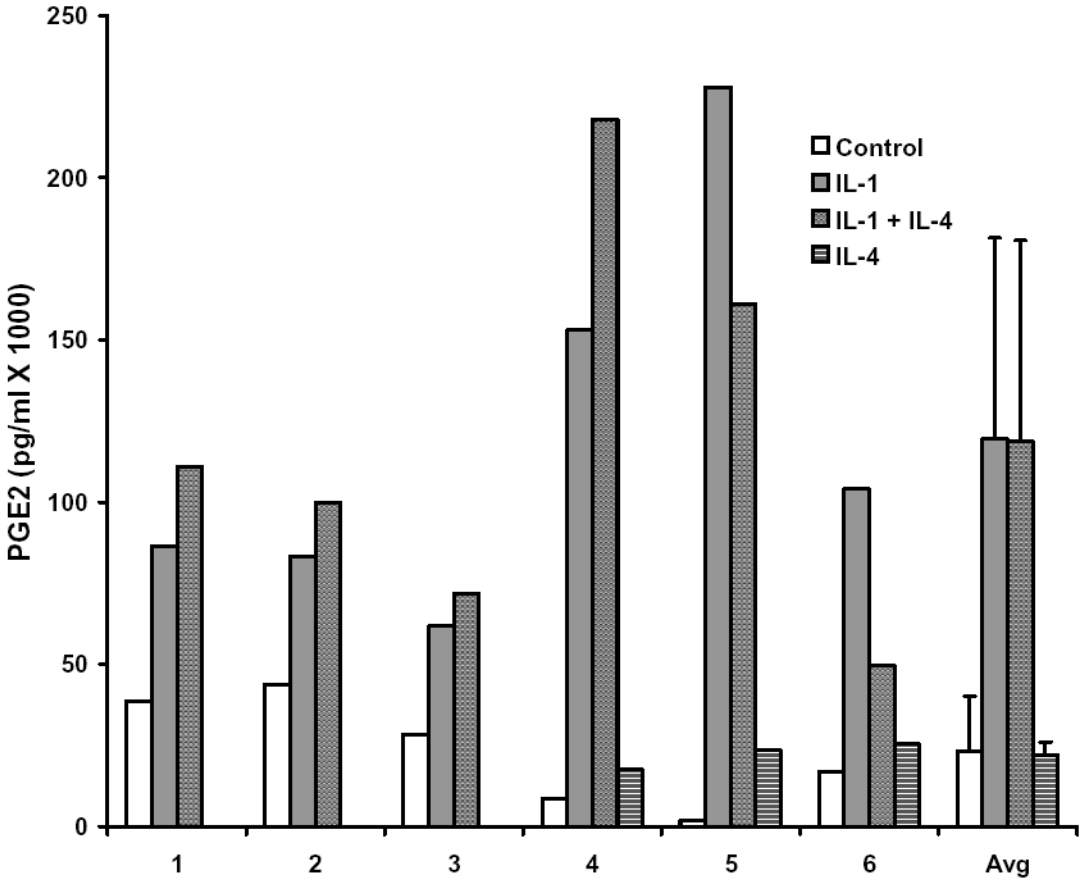

Figure 4.

Interleukin-4 (IL-4) does not inhibit IL-1 induced production of prostaglandin E2 in human gingival fibroblasts isolated from patients with periodontitis. Levels of PGE2 were measured in triplicate in conditioned medium from HGF cultures treated for 6 hours with 100 ng/ml IL-1 alone or IL-1 and 10 ng/ml IL-4. HGF cultures isolated from 6 different individuals (numbered 1 through 6 on the graph) were used.

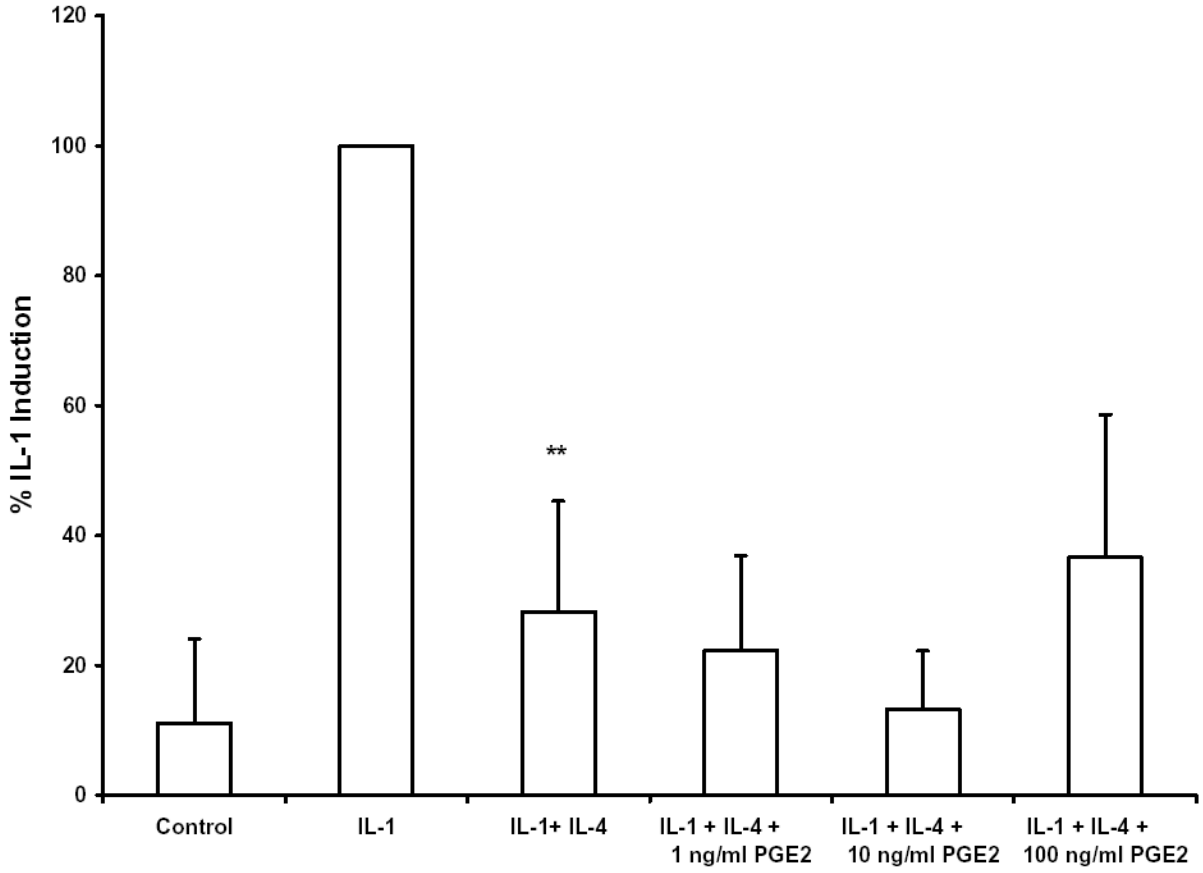

Figure 5.

Interleukin-4 (IL-4) inhibition of IL-1-induced expression of MMP-3 mRNA is independent of PGE2. Indicated amounts of PGE2 were added to cultures of HGF simultaneously with 100 ng/ml IL-1β and 10 ng/ml IL-4. Total RNA was isolated after 6 hours, and Northern blots were hybridized with cDNA probes corresponding to MMP-3 and GAPDH. Blots were quantitated by scanning densitometry and normalized to levels of GAPDH. Shown are data from seven independent experiments, utilizing HGF cultures derived from seven different donors. (** $p<0.01$ vs. IL-1 alone)

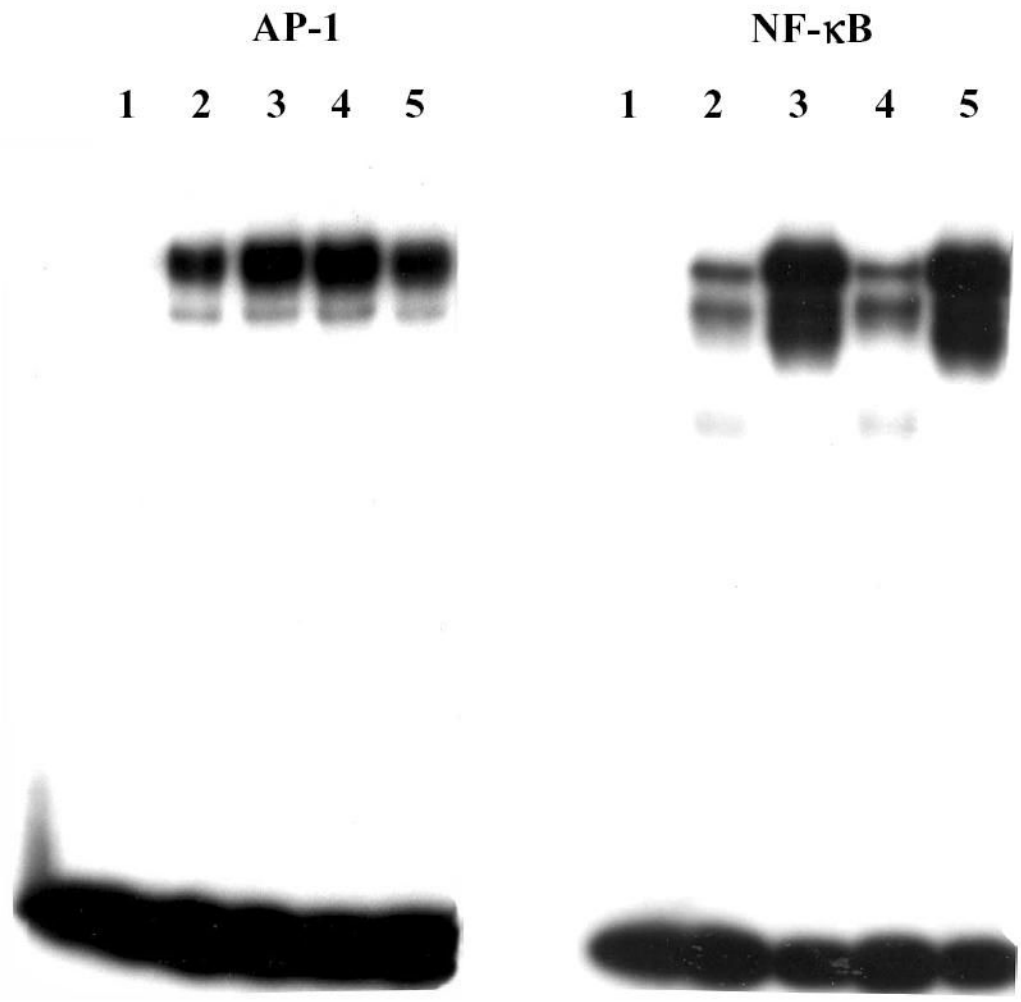

Figure 6.

DNA binding of transcription factors activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) is not affected by IL-4. Nuclear extracts were isolated from HGF cultures treated for one hour with 100 ng/ml IL-1β (lane 3), 10 ng/ml IL-4 (lane 4) or both IL-1 and IL-4 (lane 5), as well as from control cultures (lane 2). Lane 1, probe alone, no nuclear extract. Binding of 5 μg nuclear extract to ^{32}P-labeled oligo(dT) probes corresponding to consensus AP-1 and NF-κB binding sites were determined by electrophoretic mobility shift assay.