Published in final edited form as:

Exp Cell Res. 2013 June 10; 319(10): 1398–1408. doi:10.1016/j.yexcr.2013.04.010.

IL-4 inhibition of IL-1 induced Matrix Metalloproteinase-3 (MMP-3) expression in human fibroblasts involves decreased AP-1 activation via negative crosstalk involving of Jun N-terminal Kinase (JNK)

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Abstract

Matrix metalloproteinase-3 (MMP-3) over-expression is associated with tissue destruction in the context of chronic inflammation. Previous studies showed that IL-4 inhibits induction of MMP-3 by IL-1 β , and suggested that AP-1 might be involved. Here we show that IL-1 induced binding of transcription factor AP-1 to the MMP-3 promoter consists primarily of c-Jun, JunB, and c-Fos and that binding of c-Jun and c-Fos is inhibited by the combination of cytokines while binding of Jun B is not. Mutation of the AP-1 site in the MMP-3 promoter decreased the ability of IL-4 to inhibit its transcription in transfected MG-63 cells. Western blotting showed that both cytokines activate Jun N-terminal kinase (JNK), but with somewhat different kinetics, and that activation of JNK by both cytokines individually is inhibited by the combination. These results indicate that IL-4 inhibition of MMP-3 expression is associated with reduction of IL-1 induced binding of active forms of the AP-1 dimer, while less active JunB-containing dimers remain, and suggest that these changes are associated with decreased activation of JNK.

Keywords

Matrix metalloproteinase; AP-1; cJun; cFos; Interleukin 1; Interleukin 4

Introduction

Chronic inflammation in rheumatoid arthritis and periodontitis is characterized by increased levels of IL-1 β , TNF α and prostaglandin E2 (PGE2), which contribute to destruction of joints and alveolar bone in part through increased production of matrix metalloproteinases (MMPs) [1–4]. In addition, there is a relative absence of IL-4-producing T cells at sites of inflammation [5–10]. This imbalance is progressive, with decreasing levels of IL-4 correlated with loss of collagen and with increasing clinical severity [11]. 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 [12] and against collagen-induced arthritis [13]. However, a phase I/II clinical trial testing topical application of IL-4 in patients with oral squamous cell carcinoma was terminated early due to limiting toxicity [14]. It would therefore be beneficial to understand

the molecular basis of the beneficial effects of IL-4 in order to facilitate the design of therapeutics that mimic those effects, but with fewer side-effects.

MMP-3 (stromelysin-1) is a metalloproteinase with broad substrate specificity, degrading proteoglycan, laminin, fibronectin, and the non-fibrillar collagens [15,16]. It is also capable of activating other pro-MMP's, including MMP-1, -8, -9 and -13 [17–19], of inactivating plasminogen activator inhibitor I [20], and of cleaving E-cadherin [21] and FasL [22]. MMP-3 is produced by 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 [23–26], and the levels are correlated with clinical parameters and associated with progression of the disease [26]. A similar situation exists in rheumatoid arthritis [9], in which baseline serum levels of MMP-3 predict arthritis progression 8 years later [27]. IL-4 has been shown to inhibit the IL-1 induction of MMP-3 expression in human conjunctival fibroblasts [28], skin fibroblasts [29] and articular chondrocytes [30,31], as well as in human synovial [32] and gingival fibroblasts (HGF) [33,34]. Although inhibition of MMP-3 expression by IL-4 was shown to occur at the level of transcription [32], the mechanisms involved in this inhibition have not been fully elucidated.

AP-1 is a dimeric transcription factor that consists of homo- or heterodimers of members of the Jun, Fos, and ATF leucine-zipper-containing protein families. Jun proteins can form homodimers, or heterodimers with Fos or ATF proteins, but Fos proteins can only form heterodimers with Jun proteins. Different dimers may display differences in DNA binding site selection and/or affinity, as well as different activation potentials. c-Jun, c-Fos and FosB are considered strong activators, while JunB, JunD, Fra-1 and Fra-2 are relatively weaker, and may sometimes act as repressors by displacing stronger dimers [35,36].

AP-1 binding and transcriptional activity are regulated at numerous levels, including transcription, translation, mRNA and protein stability, as well as post-translational modifications of the proteins by members of the mitogen activated protein kinase (MAPK) family, including JNK, ERK and p38 MAPK. Phosphorylation by MAPKs can both stabilize and activate the proteins. In addition cJun has an AP-1 binding element in its promoter that allows autoinduction [35].

Many MMP genes, including MMP-3, have an AP-1 site at about the same location (–70) relative to the transcriptional start site, and AP-1 is important in their regulation [37]. In the case of MMP-3, this AP-1 element has been shown to be important for basal expression of MMP-3, but not sufficient for IL-1 induction [38]. Early studies suggested that AP-1 was unlikely to be involved in IL-4 inhibition of MMP-3 expression based on unchanged AP-1 binding in gelshift assays [32,39]. However, more recent experiments, including gelshift using longer DNA probes, suggested that reduced AP-1 binding and/or activity might be at least a partial explanation [34].

The present study was undertaken to study regulation and DNA binding potential of members of the AP-1 family in fibroblasts treated with IL-1 and/or IL-4, and to assess the actual binding of members of the AP-1 family to the endogenous MMP-3 promoter. Here we provide evidence that IL-4 suppression of IL-1-induced MMP-3 expression is mediated at least in part by inhibition of AP-1 binding to the MMP-3 promoter, and that this inhibition occurs on several levels, including decreased mRNA and protein expression of c-Jun, as well as decreased binding of c-Jun and c-Fos, while binding of the less-active Jun B is preserved in the presence of both cytokines. Preliminary experiments suggest that these events are associated with negative crosstalk between the two cytokines at the level of Jun N-terminal kinase (JNK) activation.

Material and methods

Cell culture

Human gingival tissue samples from patients undergoing periodontal surgery were obtained from Kevan S. Green, DDS. The tissue was processed by enzymatic dispersion to produce primary cultures. Cells were maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (penicillin, streptomycin, amphotericin; Gibco/BRL). Cells between passages 3 and 5 were used for experiments. Human Foreskin Fibroblasts (CRL2429) and MG-63 human osteosarcoma cells were obtained from the American type culture collection (ATCC) and maintained as directed. Prior to isolation of RNA or protein, HGF or HFF cells were serum-deprived for 16 h in serum free EMEM supplemented with 10% Insulin, Transferrin, Sodium selenite (Sigma Chemical Co.) prior to the addition of 10 ng IL-1 β /ml (DuPont-Merck Corp.) and/or 10 ng/ml IL-4 (Sigma Chemical Co.).

Real-time PCR

Total RNA was isolated from HGF or MG-63 cell cultures using the RNA Aqueous RNA Isolation kit for PCR (Ambion) according to manufacturer's instructions. Two mg RNA was converted to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystem) and Thermal Cycler Genius (25 °C 10 min, 37 °C 2 h, 4° hold). Two μ l of cDNA was used with 12.5 μ l of Taqman Universal PCR Master Mix (Applied Biosystem), 8.75 μ l H₂O and 1.25 μ l probes (c-Fos, c-Jun, Fra-1, JunB, MMP-3, Glyceraldehyde-3-Phosphate Dehydrogenase-GAPDH; Applied Biosystem) for Real Time PCR reactions. Real Time PCR was performed using the Applied Biosystem Prism 7000 Real Time PCR system. Reactions were done in triplicate and results were calculated according to the $\Delta\Delta$ CT method, normalized to GAPDH and expressed as percent maximal induction.

Western blotting

Whole cell or nuclear protein lysates were obtained from HGF or HFF cell cultures treated or not with IL-1 (10 ng/ml) and/or IL-4 (10 ng/ml) for indicated times using lysis buffer plus protease inhibitors from Active Motif. Samples were quantitated using a micro plate BCA protein assay kit (Thermo Scientific). Twenty or 25 μ g protein was added to 2X SDS loading buffer, boiled for 3 min and then loaded on 10% SDS polyacrylamide gels. Transfer of protein to a nitrocellulose membrane was completed using Invitrogen 7 min gel transfer system. Blots were blocked for an hour using a 5% milk/1% TBST solution before incubation overnight with the primary antibody in the same milk/TBST at 4 °C. Antibodies against human c-Jun, c-Fos, pJNK, pERK, p-p38 MAPK and β -tubulin were all obtained from Santa Cruz. Blots were washed the next morning and secondary antibody added for 1 h at room temperature. Bands were detected using Immun-Star WesternC chemiluminescent reagent (Bio-Rad) and Bio-Rad Versa Doc 4000 MP imaging system with Quantity 1 software (Bio-Rad).

AP-1 binding assay

AP-1 DNA binding activity was assessed using the Trans-Am AP-1 Transcription Factor ELISA Kit (Active Motif), which assesses DNA binding of active forms of AP-1 family proteins (c-Fos, FosB, Fra-1, c-Jun, JunB, JunD) to a consensus binding site. Twenty micrograms HGF or HFF nuclear extract were used in these experiments, which were performed according to the manufacturer's suggested protocol. Nuclear extract were isolated using the Active Motif nuclear isolation kit (ActiveMotif), and samples were quantitated using a microplate BCA protein assay kit (Thermo Scientific).

Chromatin immunoprecipitation (ChIP)

Binding of AP-1 family proteins to the AP-1 site in the endogenous MMP-3 promoter was assessed in HFF cells by chromatin immunoprecipitation using the SimpleChip Enzymatic Chromatin IP Kit (Cell Signaling and Technology) according to the manufacturer's suggested protocol. Antibodies to c-Jun, c-Fos, JunB, JunD, and Fra-1 and IgG (Santa Cruz Bio) were used for immunoprecipitation, as well as control antibodies provided with the kit. Quantitation of the immunoprecipitated DNA was performed using quantitative RT PCR. Primers specific for the region of the human MMP-3 promoter containing the AP-1 site (-231 to -101, Invitrogen) were used with a Syber Green master mix containing rox reference dye (SA Biosciences) for the PCR reaction. PCR was performed in triplicate using a 96 well plate and ABI Prism absolute quantitation software (Applied Biosystems).

Transfection

The wild-type 6TStro plasmid consists of a 2.3 kb fragment of the human MMP-3 promoter in a pGLBasic luciferase reporter plasmid, and has been used previously [40–42]. The mAP1 plasmid was acquired through site directed mutagenesis of the AP-1 site at -70 of the wild-type 6TStro plasmid (Ana-Gen Technologies). The pAP-1-Luc Cis-Reporter Plasmid was obtained from Stratagene. SV β gal was used to normalize for transfection efficiency, and pBluescript was added as needed to equalize the amount of DNA. Cells were harvested by trypsinization, washed and resuspended in 150 μ l electroporation buffer (Mirus) along with the plasmids to be transfected in electroporation cuvettes and pulsed twice for 20 ms at 110 V. DMEM was added to each cuvette and the cells were replated. Transfected cells were allowed to rest for 24 h prior to addition of IL-1 (10 ng/ml) and/ or IL-4 (10 ng/ml). Cells were harvested after 12 h and luciferase and β -gal activity were determined in the cell lysates using assay buffers from Promega or Clontech, respectively.

Results

IL-1- and IL-4-induced changes in AP-1 family mRNA and protein expression

Results of real-time PCR experiments (Fig. 1) show that expression of several members of the AP-1 family (c-Jun, JunB, c-Fos, and Fra-1) is increased by IL-1 in HGF isolated from patients with periodontitis. As was shown previously [39], IL-1 caused a large and transient increase in c-Fos mRNA levels at 0.5 h, while IL-4 had no effect on either basal or IL-1 induced expression. c-Jun mRNA was induced by IL-1 at 0.5 and 1 h, but returned to near basal levels within 3 h. IL-4 alone had no effect on c-Jun expression, but did partially inhibit the IL-1 induction at 1 h. JunB mRNA was also increased by IL-1 at 0.5 h, and then declined to basal levels within 3 h. The timing of this decline was variable between different HGF cell lines, resulting in large error bars and an overall lack of statistical significance at the 1 and 3 h timepoints. IL-4 did not inhibit IL-1 induced expression of JunB; in fact, there was a trend toward increased JunB expression in the presence of both cytokines. Fra-1 expression was also increased by IL-1, but the peak expression was smaller and occurred later as compared to c-Fos. IL-4 alone had no effect, but in combination with IL-1 it caused a trend toward decrease in Fra-1 expression.

Western blot analysis of Jun and Fos protein expression was performed in order to determine whether the cytokine-induced changes seen at the mRNA level are also seen at the protein level (Fig. 2A). The antibody directed against human c-Fos also detects FosB and Fra-1. Surprisingly, c-Fos and FosB proteins were readily detectable in whole protein lysates isolated from HGF under basal conditions, and changes induced by IL-1 were minor compared to the large and transient increase in c-Fos mRNA. IL-4 had only modest effects as well, until 3 h, when IL-4 inhibited expression of c-Fos in the presence of IL-1. FosB protein expression was similar to c-Fos, but Fra-1 and c-Jun protein results mirrored the

mRNA results more closely, with lower basal expression, IL-1 up-regulation, and partial IL-4 inhibition. The unexpected results with c-Fos were confirmed using nuclear extract and an antibody specific for c-Fos (Fig. 2B). Interestingly, in HFF normal human fibroblasts, c-Fos protein was induced by IL-1 as expected based on the RNA results (Fig. 2C).

IL-1- and IL-4-induced changes in DNA binding of AP-1 family proteins

The Trans-am AP-1 Family Transcription Factor ELISA (Active Motif) was used to determine the effects of IL-1 and IL-4, alone and in combination, on DNA binding activity of the activated forms of several AP-1 family proteins in three different HGF cell lines isolated from three different patients with periodontitis (Fig. 3). Except for JunB, which showed considerable variation, results were generally consistent among the three HGF cell lines. Surprisingly, levels of c-Fos binding were not affected by either IL-1 or IL-4, but remained at basal levels at both 1 and 3 h. Binding of the phosphorylated (active) form of c-Jun was increased by IL-1, about two-fold over basal levels at 3 h. Treatment with IL-4 caused a trend toward decreased pc-Jun binding as compared to control, and significantly inhibited the IL-1 induced binding at 3 h. JunB binding, on average, was not affected by either cytokine, alone or in combination. However, the individual cell lines differed in basal levels and IL-1 induction. Although Fra-1 binding was not significantly induced by either cytokine, IL-4 did inhibit binding relative to control, and when added in the presence of IL-1, reduced the IL-1 induction of binding.

Human foreskin fibroblast (HFF) nuclear extract was also used in the AP-1 binding assay in order to determine whether the findings in HGF were applicable to fibroblasts in general. Interestingly, the basal levels of pc-Jun and Fra-1 binding seemed to be higher in the “inflamed” gingival cells as compared to “normal” HFF. Binding of pc-Jun was induced by IL-1 and inhibited by IL-4 at both 1 and 3 h, but the levels of binding were never more than half of that detected in HGF. Binding of c-Fos was similar in HGF and HFF under basal conditions. Although there was a substantial increase in c-Fos binding by both IL-1 and IL-4 in HFF, when the cytokines were combined, IL-4 seemed to inhibit the IL-1 induction at 1 h. JunB binding in HFF was similar to HGF in that no significant differences were detected. Fra-1 DNA binding in HFF showed a trend toward induction by both cytokines, but a trend toward inhibition by the combination did not reach statistical significance.

IL-1- and IL-4-induced changes in binding of AP-1 family proteins to the endogenous MMP-3 promoter

Although the results of the AP-1 binding assay were informative, changes in binding to a consensus oligonucleotide in vitro may not accurately reflect changes in binding to an actual promoter in its normal chromatin configuration. Chromatin immunoprecipitation (ChIP) was performed in HFF in order to avoid the variation that is sometimes seen with HGF.

In untreated cells, very little binding of AP-1 proteins was detected, similar to IgG negative controls. Fig. 4 shows binding of c-Jun, c-Fos, JunB and Fra-1 relative to control, at 1 and 3 h. After 1 h, relatively little change was seen with IL-1 treatment, whereas IL-4 induced binding of JunB. At 3 h, IL-1 induced c-Jun, JunB, and c-Fos binding. IL-4-induced binding of JunB was transient, decreasing by 3 h. IL-4 also induced binding of c-Jun (more than IL-1) and c-Fos (much less than IL-1). When IL-1 and IL-4 were combined, c-Jun and c-Fos binding was markedly diminished relative to induction by either cytokine alone, and JunB binding was reduced compared to IL-1 alone. Fra-1 binding was comparable to IgG under all conditions. Thus the combination of cytokines decreased binding of c-Jun and c-Fos proteins to the MMP-3 promoter compared to either cytokine alone, while binding of JunB was maintained at near the same level as IL-4 alone.

Role of the AP-1 site in IL-4 inhibition of MMP-3 transcription

In order to establish more firmly the role of AP-1 in IL-4 inhibition of MMP-3 expression, transient transfections were carried out in MG-63 cells using luciferase reporter constructs containing a 2.3 kb fragment of either the wild-type human MMP-3 promoter or the same fragment mutated at the AP-1 site. Preliminary experiments confirmed that MMP-3 expression is induced by IL-1 and inhibited by IL-4 in these cells (Fig. 5A). Results of transfection experiments (Fig. 5B) showed that mutation of the AP-1 site decreased both basal and IL-1-induced transcription, as well as IL-4 inhibition. An AP-1 reporter plasmid confirmed that IL-4 inhibits basal and IL-1 induced transcriptional activity (Fig. 5C).

Activation of mitogen activated protein kinases in response to IL-1 and IL-4

Regulation of AP-1 binding and transcriptional activity is quite complex, and involves post-translational modification by members of the MAPK family: Jun N-terminal Kinase (JNK), ERK, and p38 MAPK [35]. In order to determine which of these kinases is activated (phosphorylated) in response to IL-1, IL-4 or the combination in HGF, Western blotting was performed. Results are shown in Fig. 6. In HGF, all three kinases were at least somewhat active under basal conditions in the absence of cytokines. Both IL-1 and IL-4 increased levels of phospho-JNK, but with different kinetics. IL-1 caused early and transient activation at 15 min, while activation by IL-4 is seen at 30 min. When the two cytokines are combined, both activations are inhibited. Levels of phosphorylated forms of p38 MAPK and ERK were relatively unchanged during this timeframe. In HFF (Fig. 6B), the activation pattern of JNK was similar to that seen in HGF, while p-p38 and p-ERK showed lower basal levels, some IL-1 induction, and perhaps a slight decrease in the presence of both cytokines as compared to IL-1 alone.

Discussion

MMP-3 expression is induced by inflammatory cytokines and high levels are associated with tissue destruction in the context of chronic inflammation such as occurs in periodontal disease and rheumatoid arthritis. In particular, IL-1 induces MMP-3 expression in a variety of cell types and IL-4 has been shown to inhibit the IL-1 induction of MMP-3 expression in human conjunctival fibroblasts [28], skin fibroblasts [29] and articular chondrocytes [30,31], as well as in synovial [32] and gingival fibroblasts (HGF) [33,34]. Although IL-4 has been shown to inhibit AP-1 activity in other systems [43,44], initial gelshift experiments in synovial and gingival fibroblasts failed to demonstrate decreased AP-1 binding in the presence of IL-4 [32,39] until a longer deoxynucleotide probe was used [34]. The current series of experiments was undertaken to determine whether and how IL-4 inhibition of AP-1 activity is involved in its ability to inhibit the IL-1 induced expression of MMP-3 in human fibroblasts.

c-Jun mRNA and protein expression, as well as DNA binding, were consistently increased by IL-1 and decreased by IL-4 in both HGF and HFF, though the magnitude of these effects was not large. In addition, its binding to the endogenous MMP-3 promoter was similarly activated and inhibited. Since c-Jun-containing dimers are strong activators of transcription [35], these changes are consistent with a role for inhibition of AP-1 binding in IL-4's ability to suppress IL-1 induced MMP-3 expression.

c-Fos mRNA induction by IL-1 was substantial and transient and unaffected by the presence of IL-4, as previously reported [32]. The relatively minor changes in c-Fos protein expression in HGF were quite surprising and could reflect inefficient translation (possibly due to the presence of a microRNA that inhibits its translation [45]), or instability of the c-Fos protein. c-Fos protein is stabilized by phosphorylation of its C-terminus by ERK, and by

formation of dimers with Jun proteins [46]. Since active ERK was easily detectable in HGF (Fig. 6), it is more likely that c-Fos protein would be unstable due to relatively lower levels of Jun proteins with which to dimerize. Consistent with that hypothesis are the relatively higher levels of Fra-1 binding activity. It is possible that Jun/Fra-1 dimer formation is preferred when Fra-1 is available, leaving the c-Fos protein to rapidly degrade without a binding partner. It is also possible that the Jun/Fra-1 dimer competes more effectively than Jun/c-Fos dimers for binding to the consensus site immobilized on the assay plate. This would also decrease the relative amounts of c-Jun binding detected by the assay. In HFF, however, where basal levels of Fra-1 binding are lower than in HGF (but still induced by IL-1 and inhibited by IL-4), binding of c-Fos both in the in vitro binding assay and in ChIP was dramatically induced by IL-1 and inhibited by IL-4. Again these results are consistent with a role for AP-1 in IL-4 inhibition of MMP-3 expression.

In addition to decreases in binding of AP-1 dimers containing c-Jun and cFos, IL-4 also increased levels of JunB, and JunB binding to the MMP-3 promoter was still evident when the two cytokines were combined. Since JunB is considered at best a weak activator of transcription, this too is consistent with decreased AP-1 activity at the MMP-3 promoter in the presence of IL-4.

It is interesting to note that the basal levels of c-Jun and Fra-1 binding seemed to be higher in HGF as compared to HFF, with Jun B showing a similar but less dramatic trend. This difference could be related to the different tissues of origin (gingival vs. dermal), to the difference in ages of the tissue donors (infant vs adult), or to the chronic exposure of HGF to inflammatory stimuli. Ebisawa et al. [47] compared low passage-number normal gingival fibro-blasts to normal dermal fibroblasts from donors of same age, and found them to be nearly identical in terms of their fundamental characteristics, morphology and function. However, there were some differences, with about 5% of genes being uniquely expressed, and some of those were said to be related to cytokine responses. In support of the latter interpretation is the fact that, although this constitutive activation of AP-1 has not been previously demonstrated in periodontitis derived HGF, it is well established in rheumatoid arthritis, another chronic inflammatory condition. AP-1 DNA binding activity is markedly up-regulated in synovial cells derived from patients with rheumatoid arthritis as compared to osteoarthritis, and this has been correlated with disease severity [48] and with “tumor-like” growth of synovial cells [49].

The ChIP assay utilized HFF in order to minimize variability sometimes seen between different HGF cultures, and examined which members of the AP-1 family actually interact with the MMP-3 promoter following exposure to IL-1, IL-4 or the combination for 1 or 3 h. As expected from the binding assay, basal levels of AP-1 binding were very low. Dimers binding to the MMP-3 promoter in response to IL-1 might include c-Jun/c-Fos and JunB/c-Fos, as well as c-Jun and JunB homodimers. The c-Jun/c-Fos dimers would be expected to be the most transcriptionally active. IL-4 induces binding of the same proteins, but in different proportions. Although c-Fos binding is induced by IL-4, the magnitude is much less than induced by IL-1, so dimers binding in the presence of IL-4 are more likely to include homodimers and/or heterodimers of c-Jun and/or JunB. In the presence of both cytokines, only JunB binding is detected. Since JunB is less transcriptionally active than c-Jun [35], the ChIP results support the hypothesis that changes in AP-1 binding and dimer composition contribute to the decreased transcription of MMP-3 by IL-4. Further support comes from transient transfection of an MMP-3 reporter construct in which mutation of the AP-1 binding site significantly reduces the magnitude of IL-4 inhibition of transcription in the presence of IL-1, as well as an AP-1 reporter plasmid, showing decreased AP-1 dependent transcription in the presence of IL-4 (Fig. 5).

As a first step toward determining the mechanism of IL-4 effects on AP-1 proteins, the levels of phosphorylated (active) members of the MAPK family were examined by western blotting. Active forms of all three major MAPKs known to act on AP-1 were readily detected in HGF, even under basal conditions. In HFF, basal levels of p-p38 and p-ERK seemed lower, with more IL-1-induced activation. This is consistent with the hypothesis that HGF cells have an “activated” phenotype, and might explain the high basal levels of AP-1 protein and binding detected in these cells. Interestingly, only pJNK levels seemed to be affected by treatment with cytokines in HGF. Both cytokines increased levels of active JNK, but combination of the two diminished both inductions. IL-1 induced activation of JNK was also inhibited by IL-4 in HFF cells. JNK activates c-Jun by phosphorylating it on Ser 63/73 and Thr 91/93, which increases protein stability, DNA binding and transcriptional activity. The effects on AP-1 activity are further increased by the fact that c-Jun auto-regulates by binding to an AP-1 site in its own promoter [51]. These results suggest that inhibition of IL-1-induced MMP-3 expression by IL-4 involves negative cross-talk at the level of JNK activation, followed by decreased AP-1 binding and alteration of the dimer to a less active form. In addition, these findings support the rationale for development of JNK and/or AP-1 inhibitors to help maintain or restore an appropriate balance between “inflammatory” and “anti-inflammatory” responses.

Acknowledgments

This work was supported in part by grant R15DE16277 from the NIDCR to R.C. Borghaei.

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Fig. 1.

Effects of IL-1 and IL-4 on Jun and Fos mRNA expression HGF cell cultures were serum-deprived for 16 h prior to the addition of 10 ng IL-1 β /ml and/or 10 ng/ml IL-4. Total RNA was harvested from control (untreated) cells and from cells treated for 0.5, 1 or 3 h with cytokine(s). c-Jun, JunB, c-Fos, and Fra-1 mRNA levels were quantitated by real-time PCR and normalized to levels of GAPDH mRNA. The graph represents data from three independent experiments with three different HGF cultures derived from samples from three different individuals, average $\pm$ SEM. Statistical significance was assessed by ANOVA followed by Tukey's post-hoc test.

Fig. 2.

Effect of IL-1 and IL-4 on Jun and Fos protein expression (A) HGF cell cultures were serum-deprived for 16 h prior to the addition of 10 ng IL-1 β /ml and/or 10 ng/ml IL-4. Whole cell protein lysates were obtained from control (untreated) cells and from cells treated for 30 min, 1 h or 3 h with cytokine(s). Twenty μ g of each lysate was separated on 10% SDS-PAGE and transferred to a nylon membrane. Proteins were detected with anti-c-Fos antibody, which also recognizes Fra-1 and FosB, as well as anti-c-Jun and β -tubulin antibodies, followed by chemiluminescence. (B and C) HGF and HFF cell cultures were serum-deprived for 16 h prior to the addition of 10 ng IL-1 β /ml and/or 10 ng/ml IL-4. Nuclear extracts were isolated from control (untreated) cells and from cells treated for 30 min or 3 h with cytokine(s). Twenty-five μ g of each extract was separated on 10% SDS-PAGE and transferred to a nylon membrane. Proteins were detected with anti-c-Fos antibody specific for c-Fos, as well anti- β -tubulin antibody, followed by chemiluminescence.

Fig. 3.

Effect of IL-4 on IL-1 induced binding of AP-1 family proteins to a consensus AP-1 site. HGF or HFF cultures were serum-deprived for 16 h and then incubated for 0.5, 1 or 3 h with IL-1 (10 ng/ml) and/or IL-4 (10 ng/ml). Control cells were incubated without cytokine addition. AP-1 DNA binding activity was determined using the TransAM AP-1 Family ELISA-based binding assay from Active Motif and 20 μ g nuclear extract. For HGF, data shown are from three independent experiments with three different HGF cultures derived from samples from three different individuals, average $\pm$ SEM. Statistical significance was assessed by ANOVA followed by Tukey's post-hoc test.

Fig. 4.

Effect of IL-4 on IL-1 induced binding of AP-1 proteins to the endogenous MMP-3 promoter HFF cells were incubated in the presence and absence of IL-1 and/or IL-4 for 1 or 3 h. ChIP assays were performed using the SimpleChip Enzymatic Chromatin IP Kit from Cell Signaling and Technology according to the manufacturer's suggested protocol. Antibodies to c-Jun, c-Fos, JunB, JunD, and Fra-1 and IgG were used for immunoprecipitation. Quantitation of the immunoprecipitated DNA was performed by quantitative RT PCR using primers specific for the region of the human MMP-3 promoter containing the AP-1 site (-231 to -101). Results are expressed as percentage of input relative to the untreated control.

Fig. 5.

Effect of mutation of the AP-1 site on IL-4 inhibition of transcription from the MMP-3 promoter (A) Total RNA was harvested from control (untreated) MG-63 cells and from cells treated for 12 h with 10 ng IL-1 β /ml and/or 10 ng/ml IL-4. MMP-3 mRNA levels were quantitated by real-time PCR and normalized to levels of GAPDH mRNA. The graph represents data from three independent experiments, average $\pm$ SEM. (B) MG-63 cells were transfected with a luciferase reporter plasmid containing 2.3 kB of the wild-type human MMP-3 promoter (WT), or with a similar plasmid containing a mutation in the AP-1 site at -70 (mAP-1), or (C) an AP-1 reporter plasmid, along with SV β gal as a control for transfection efficiency. Cells were replated and allowed to rest for 24 h prior to addition of IL-1 (10 ng/ml) or IL-1+IL-4 (10 ng/ml). Cells were harvested after 12 h. Normalized luciferase numbers were expressed as % inhibition of the IL-1+IL-4 combination relative to IL-1 alone. Statistical significance was assessed using Student's *T*-test.

Fig. 6.

Effect of IL-1 and IL-4 on levels of activated forms of MAPKs. (A) HGF or (B) HFF cell cultures were serum-deprived for 16 h prior to the addition of 10 ng IL-1β/ml and/or 10 ng/ml IL-4. Whole cell protein lysates were obtained from control (untreated) cells and from cells treated for indicated times with cytokine(s). Twenty μg of each lysate was separated on 10% SDS-PAGE, and transferred to nylon membrane. Proteins were detected with antibodies directed against the activated (phosphorylated) forms of JNK, ERK and p38 MAPK, as well as β-tubulin.