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Differential regulation of metalloprotease steady-state mRNA levels by IL-1 and FGF in rabbit articular chondrocytes

Srinivasan Chandrasekhar and Anita K. Harvey

Skeletal Diseases Research, Lilly Research Laboratories, Indianapolis, IN 46285, USA

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The expression of collagenase and stromelysin is believed to be coordinately regulated. In this report however, we provide evidence that suggests subtle differences may exist in the early events of the induction of these enzymes. Rabbit articular chondrocytes treated with interleukin-1, either alone or in combination with fibroblast growth factor, accumulated steady-state mRNA levels for both the enzymes, with the latter treatment more effective in inducing greater levels and within a shorter time. Further, the induction of the enzymes by either protocol was blocked by cycloheximide co-treatment. Cycloheximide added 1 h post-stimulation with interleukin-1 + fibroblast growth factor failed to block stromelysin mRNA expression, but was able to block collagenase steady-state mRNA levels. Transforming growth factor-β, another inhibitor of metalloprotease induction, showed no such differential activity. The results suggest that collagenase and stromelysin may have subtle variations in their induction pathways. Our studies further show that the enzyme induction by interleukin-1 alone or in combination with fibroblast growth factor occurs through different, but related mechanisms.

Metalloprotease regulation; Collagenase; Stromelysin; Cytokine; Interleukin-1; Cartilage destruction; Fibroblast growth factor

1. INTRODUCTION

Metalloproteases are believed to play an important role in cartilage matrix remodeling and destruction during development and in disease conditions such as osteoarthritis [1–4]. In general, the metalloproteases are not constitutively produced but are induced by signals such as interleukin (IL)-1 and growth factors [1,2,5–11]. Although, these enzymes are thought to be co-ordinately regulated, we provide evidence that at least 2 enzymes, collagenase and stromelysin, may display subtle differences in their expression in rabbit articular chondrocytes. In this system, only IL-1 was able to induce collagenase/stromelysin production, whereas growth factors modulated the IL-1 activity. These growth factors, when added alone, did not induce any enzyme synthesis [12–15]. The metalloproteases are synthesized as precursor proteins and require activation [1–4], but very little is known about the early post-receptor events that lead to the induction of the enzymes. We were particularly interested in understanding the similarities and differences between the induction of metalloproteases by IL-1, either alone or in combination with fibroblast growth factor (FGF). The results of the present study suggest that (i) the enzyme induction by IL-1 vs. IL-1+FGF occurs via distinct but related mechanisms, and (ii) subtle differences may exist between collagenase and stromelysin induction by IL-1+FGF.

Abbreviations: IL-1, interleukin-1; FGF, fibroblast growth factor; TGF-β, transforming growth factor-β; DMEM, Dulbecco's Modified Eagle's Medium; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Correspondence address: S. Chandrasekhar, Skeletal Diseases Research, Lilly Research Laboratories, Indianapolis, IN 46285, USA. Fax: (1) (317) 276 9722.

2. MATERIALS AND METHODS

2.1. *Growth factors and cytokines*

Recombinant human IL-1β was isolated by cloning and expression of a synthetic gene [16]. The following products were purchased: porcine platelet transforming growth factor β (TGF-β_1) (R & D Systems, Minneapolis, MN), recombinant bovine basic FGF (Amgen, CA). All treatments were carried out in serum-free Dulbecco's Modified Eagle'sMedium (DMEM) (Gibco).

2.2. *Cells and cell culture*

Chondrocytes were isolated from the tibial and femoral cartilage of 1–1.5 kg NZW rabbits [8,12] by sequential digestion with hyaluronidase (Sigma; St. Louis), TPCK-trypsin and collagenase (Worthington, Freehold, NJ). Cells were seeded at a density of 2×10^4/cm^2 in Ham's F-12 (Gibco, Grand Island, NY) medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 50 μg/ml gentamicin, were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air and were fed on days 3 and 6 after plating. For all experiments, only confluent primary cells were used within 7–10 days of culture.

2.3. *Determination of collagenase and stromelysin steady-state mRNA levels*

Chondrocytes were treated with IL-1 (30 ng/ml), TGF-β (5 ng/ml), and FGF (10 ng/ml) or a combination thereof, in serum-free DMEM for indicated time intervals. The concentrations of the cytokines and growth factors were established based on our previous observations [12,15]. The total cellular RNA was extracted with guanidine hydrochloride, then with chloroform + *n*-butanol, followed by 2 ethanol

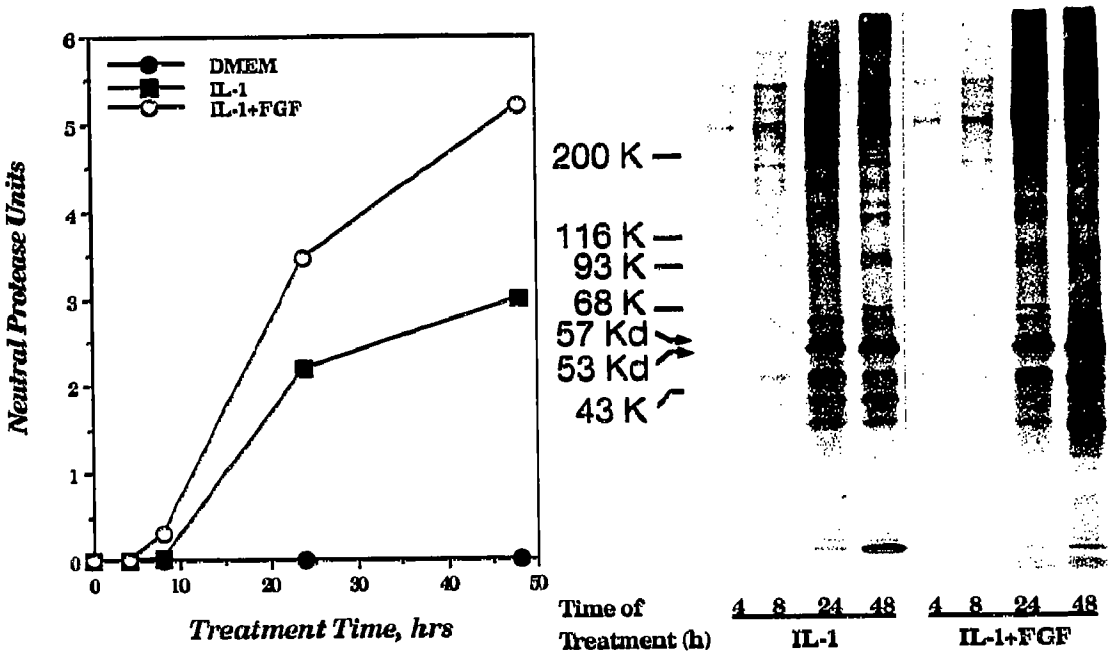

Fig. 1. Induction of neutral protease activity and synthesis by IL-1 and IL-1+FGF. Confluent monolayers of rabbit articular chondrocytes were treated with IL-1 (30 ng/ml) or FGF (10 ng/ml) or both for indicated time intervals in serum-free DMEM. The conditioned media samples were assayed (in quadruplicate) for neutral protease activity using [^{3}H]casein as the substrate (Materials and Methods). In a parallel experiment, cells were also treated with [^{35}S]methionine (40 μCi/ml) and the conditioned media were dialyzed, lyophilized and subjected to SDS-PAGE (3% stacking gel and 3–15% linear gradient resolving gel), followed by fluorography. The relative molecular weights were determined by comparing against high molecular weight standards from Bio-Rad. Cells treated with FGF alone did not induce any protease (data not shown; [12]).

precipitations [17,18]. The specific mRNAs were analyzed by either Northern blot or slot-blot by hydridizing for 18 h with oligonucleotide probes for rabbit procollagenase (nucleotide sequence 842–886) [19] and proactivator/stromelysin (877–921) [20]. The oligonucleotides were 5'-end labeled with ^{32}P using T4 polynucleotide kinase, and the signal intensity was standardized by probing the filters with 5'-end labeled oligo(dT) [21]. In experiments involving cycloheximide (Sigma) treatment, cells were treated with various combinations of IL-1 and growth factors at a constant concentration of cycloheximide (10 μg/ml). In all these experiments, cycloheximide was added either at the time of induction with IL-1 (± FGF) or at various time points after the addition of the factors, and the experiments were terminated 8 h post-stimulation with IL-1 (± FGF). The bands from autoradiograms were scanned using a laser densitometer (LKB) and the relative levels were expressed as a percentage of control values.

2.4. *Determination of neutral protease activity*

Conditioned media obtained after treatment with IL-1 ± growth factors were assayed for the neutral protease activity as described previously [8,12]. The media were incubated for 10 min at room temperature with TPCK-Trysin (100 μg/ml in a buffer containing 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 10 mM calcium acetate) to activate latent enzymes, followed by a 30 min treatment with soybean trypsin inhibitor (150 μg/ml in the same buffer). [^{3}H]Casein was reacted with the medium at 37°C for 2 h, the samples were cooled to 4°C, and the non-digested casein was precipitated by 10% (w/v) trichloracetic acid. The radioactivity in the supernatant fraction was determined, and enzyme activity was calculated by defining one enzyme unit as that amount required to digest 1 μg of substrate/min at 37°C.

2.5. *Determination of collagenase and stromelysin synthesis*

The metalloprotease synthesis was also studied by analyzing the [^{35}S]methionine (40 μCi/ml of Tran^{35}S-label, ICN) labeled conditioned media of cells subjected to various treatments by SDS-PAGE using a 3% stacking gel and a 3–15% linear gradient resolving gel [12]. High molecular weight protein standards (Bio-Rad) were included for comparison of electrophoretic mobility. The gel was equilibrated in ENTENSIFY (NEN), dried by vacuum and exposed to an X-ray film (X-Omat AR, Kodak) at −70°C.

3. RESULTS

3.1. *Neutral protease induction by IL-1 and IL-1+FGF*

Initially, we compared the level of neutral protease activity and synthesis after treatment with IL-1 or IL-1+FGF for various times. Rabbit articular chondrocytes in monolayer were treated with IL-1 (30 ng/ml), FGF (10 ng/ml) or a combination of both for the indicated time intervals, and the conditioned media were assayed for neutral metalloprotease activity. The data (Fig. 1, left panel) reveal a time-dependent increase in neutral protease activity in response to treatments with either IL-1 or IL-1+FGF. No protease activity was detectable at 4 or 8 h after IL-1 treatment, with increasing activity at 24 and 48 h. In IL-1+FGF treated cells, only a barely detectable level was found at 8 h, but higher levels were found at 24 and 48 h. At both the latter time points, IL-1+FGF-treated cells secreted greater levels of the activity than the corresponding treatment with IL-1 alone. In confirmation of our previous studies, FGF alone did not induce any protease activity [12].

These observations were further confirmed by analyzing the newly synthesized protein products. Cells

were treated as above and were also incubated with [^{35}S]methionine for the duration of the assay. The conditioned media were analyzed by SDS-PAGE (3% stacking gel and 3–15% linear gradient resolving gel). The results are displayed in Fig. 1, right panel. The protein band of 57 kDa has previously been shown to be procollagenase, whereas the 53 kDa band is likely to be prostromelysin [12]. The results further confirm the time-dependent increase in the level of the 2 protein bands. These proteins were not readily detectable up to 8 h and were clearly present at 24 and 48 h. At these time points, IL-1+FGF-treated cells contained greater levels of these protein bands.

3.2. *Time-course of metalloprotease mRNA induction by IL-1 and IL-1+FGF*

We also wanted to examine if the time-dependent effects of IL-1 and IL-1+FGF were reflected in the steady-state mRNA for the enzymes. The steady-state mRNA levels were determined by probing with oligonucleotide probes for prostromelysin and procollagenase [19,20]. Initially, we established whether the oligonucleotide probes recognized and hybridized to the appropriate metalloprotease mRNAs induced by IL-1 in rabbit articular chondrocytes. Cells were treated with IL-1 (30 ng/ml), FGF (10 ng/ml), or a combination of both for 18 h, the total RNA was extracted and analyzed by Northern blot using specific oligonucleotide probes for procollagenase and prostromelysin. The results show that cells treated with IL-1 (Fig. 2, lanes 2) or IL-1+FGF (lanes 1) contained mRNA transcripts (1.8 and 2.2 kb) which are consistent with reported sizes for prostromelysin and procollagenase, respectively [14,19,20]. They also confirm that IL-1+FGF-treated cells accumulated greater levels of both

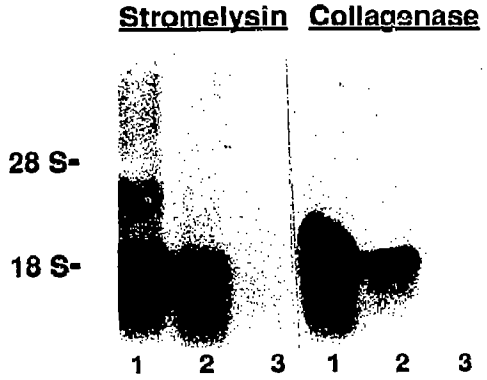

Fig. 2. Northern blot analysis of stromelysin and collagenase mRNA from rabbit chondrocytes. Articular chondrocytes were treated with (30 ng/ml) of IL-1 (lanes 2) or IL-1+FGF (30 and 10 ng/ml, respectively; lanes 1) for 18 h in serum-free DMEM. Control cells (lanes 3) were treated with DMEM alone. The total RNA was extracted, equal amounts of RNA (7 μg/lane) were fractionated on an agarose gel, stained with ethidium bromide to locate the ribosomal RNA, transferred to Genescreen and hybridized with oligonucleotide probes for stromelysin and collagenase. The hybridized bands were visualized by fluorography.

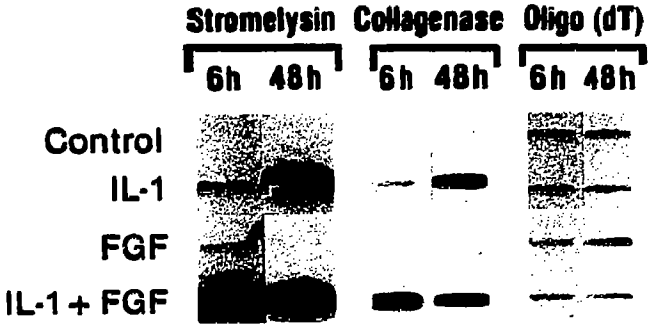

Fig. 3. Kinetics of induction of stromelysin and collagenase by IL-1 and IL-1+FGF. Chondrocytes were treated with IL-1 (30 ng/ml) or FGF (10 ng/ml) or both for 6 or 48 h, the total RNA was extracted, applied by slot-blotting onto Genescreen (2 μg/track) and probed as in Fig. 2. Another filter similarly prepared was hybridized with oligo(dT).

the mRNAs, while control cells (lanes 3) contained no detectable levels of either mRNA [12–14]. These results, taken together with the activity and synthesis data, suggest that a lag-time may exist before the translation of the induced mRNA. In order to determine the early events in the metalloprotease induction, for subsequent studies, only steady-state mRNA levels were measured.

We next asked whether collagenase and stromelysin displayed differences in the time of induction after stimulation. Chondrocytes were treated with IL-1 or IL-1+FGF for either 6 or 48 h and the specific mRNA-levels were determined by Northern hybridization after slot-blot. The data (Fig. 3) show that IL-1 induced only a low level of both collagenase and stromelysin mRNA levels 6 h post-stimulation and much higher levels at 48 h. In contrast, IL-1+FGF-treated cells showed a dramatic increase in mRNA levels within 6 h of treatment with no further increase a 48 h. Densitometric scanning of the slots confirmed these observations (not shown). Determination of total RNA applied to the slots using an oligo dT nucleotide probe indicated that comparable amounts were present in all tracks. The results suggest that IL-1+FGF is likely to cause a faster induction of collagenase and stromelysin genes by influencing events occurring within the first 6 h of stimulation. They further show that both collagenase and stromelysin displayed comparable patterns of response to the inducing signals.

3.3. *Stromelysin mRNA requires early protein synthesis*

We next evaluated whether the collagenase and stromelysin induction required new protein synthesis, and whether the new protein synthesis occurred at a specific time after stimulation. Chondrocytes were stimulated with either IL-1 of IL-1+FGF and cycloheximide was added at 0, 0.5, 1, 2 and 4 h after induction. The experiments were terminated 8 h after the treatments began.The results (Fig. 4, bottom panel) show that (i) cycloheximide co-treatment with IL-1 (tracks a) or IL-1+FGF(tracks b) for the duration of the study resulted

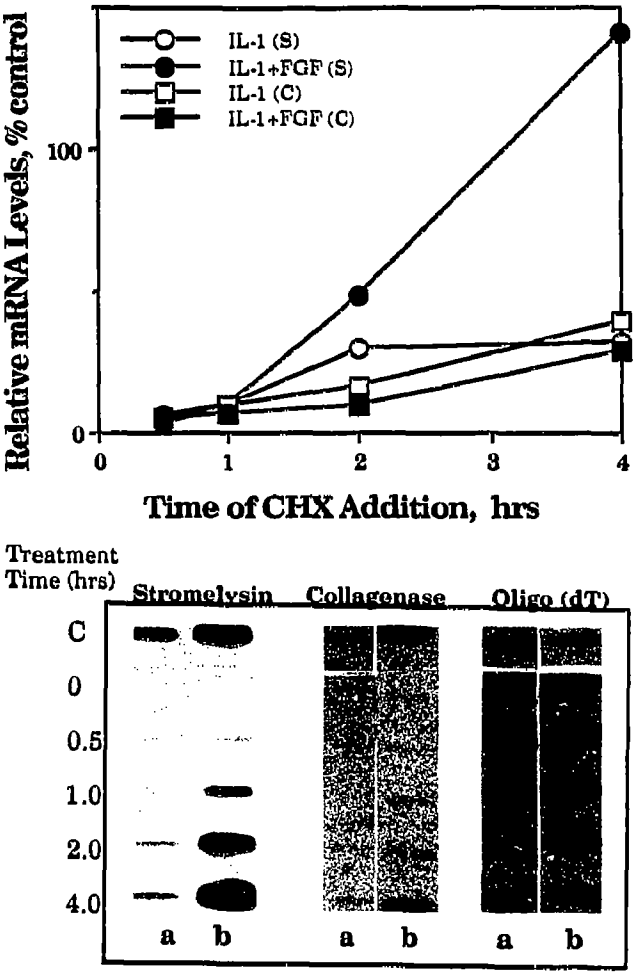

Fig. 4. Cycloheximide inhibition of collagenase and stromelysin mRNA levels. Cells were treated with IL-1 (30 ng/ml; tracks a) or IL-1 (30 ng/ml)+FGF (10 ng/ml; tracks b), then cycloheximide (10 μg/ml) was added at the same time (time 0) or 0.5, 1, 2 and 4 h later, and at 8 h post-stimulation, total RNA was extracted and the specific mRNAs were determined by hybridization with [^{32}P] end-labeled oligonucleotide probes (bottom panel). For comparison, a similar preparation of the filters was also probed with [^{32}P]oligo(dT). Control (C) cells were treated with IL-1 or IL-1+FGF for 8 h. The intensity of the bands was quantitated by scanning them by a laser densitometer, and the data are expressed as percentages of control values (top panel; S, stromelysin; C, collagenase).

in complete inhibition of both the mRNAs, suggesting that new protein synthesis was required; (ii) the induction of collagenase and stromelysin mRNA by IL-1 was blocked by cycloheximide even when it was added 4 h after stimulation, suggesting a continued requirement for new protein synthesis; (iii) cycloheximide was able to block or reduce stromelysin mRNA expression in IL-1+FGF-treated cells, only when it was added within 1 h after the stimulation. At 2 h (and beyond) post-stimulation with IL-1+FGF, no inhibition was observed. The results suggest that FGF is likely to induce factors within 1 h of addition that may be required for stromelysin mRNA synthesis; and (iv) cycloheximide blocked the accumulation of collagenase mRNA at all time points, irrespective of the method of induction. Quantitative analysis of the slots by densitometric scanning confirmed these observations (Fig. 4, top panel). Thus, the time-dependent inhibition by cycloheximide is specific to stromelysin and not to collagenase. These observations suggest that: (i) different protein(s)/factor(s) may be induced by IL-1 vs. IL-1+FGF that could play a role in stromelysin expression; and (ii) collagenase and stromelysin show subtle differences in the control mechanisms leading to the mRNA synthesis.

3.4. *Differential modulation of collagenase and stromelysin expression by cycloheximide and TGF-β*

In order to further characterize the differential effects of cycloheximide on IL-1+FGF inducible collagenase and stromelysin mRNA levels, we next compared the effects of another inhibitor of metalloprotease gene expression, namely, TGF-β, with that of cycloheximide [21,22]. Chondrocytes were stimulated with IL-1+FGF and then TGF-β or cycloheximide were added at indicated times post-stimulation. As before (Fig. 4), cycloheximide displayed a time-dependent inhibitory effect on stromelysin (Fig. 5, tracks a), but not collagenase (Fig. 5, tracks b) expression. However, TGF-β showed no difference in its ability to inhibit the accumulation of stromelysin and collagenase. TGF-β was effective only if it was added within the first 1 h of stimulation by IL-1+FGF. These results further suggest that the control of stromelysin and collagenase involves subtle differences in their pathways of induction, and that TGF-β and cycloheximide may regulate different factors that influence the expression of the 2 metalloprotease genes.

4. DISCUSSION

Tissue destruction and remodeling occur during inflammatory conditions and are likely to be influenced by a complex interplay of cytokines, growth factors and

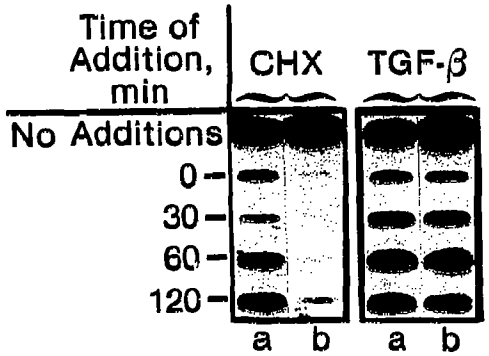

Fig. 5. Relative effects of cycloheximide and TGF-β on stromelysin and collagenase mRNA levels. Chondrocytes were treated with IL-1+FGF (30 + 10 ng/ml, respectively) and the inhibitors (cycloheximide at 10 μg/ml; TGF-β at 5 ng/ml) were added at the same time (0) or 30, 60 and 120 min post-stimulation. The total RNA was extracted and analyzed. (a) Stromelysin; (b) Collagenase.

other mediators. These mediators allow cells to elaborate metalloproteases that may play a role in the destruction of the functional extracellular matrix. It is therefore important to understand the sequence of events that lead to the induction of the proteases and whether subtle differences exist in different methods of induction. In this communication, we have compared the induction of collagenase and stromelysin mRNA by IL-1 and IL-1+FGF. We show that IL-1-treated chondrocytes accumulate increasing amounts of mRNA for stromelysin and collagenase over a 48-h period, whereas a combination of IL-1+FGF induces peak levels of the same mRNAs within 6 h. We further show that stromelysin and collagenase display subtle differences in their temporal expression in the presence of cycloheximide and TGF-β, indicating different regulatory requirements.

The time-dependent increase in the metalloprotease in response to IL-1 or IL-1+FGF was confirmed by determination of neutral protease activity, synthesis and mRNA levels. Of the 3 procedures, however, only mRNA levels were detectable within the first 6 h of induction. Since we were interested in early events that control metalloprotease expression, we evaluated only mRNA levels for stromelysin and collagenase.

In rabbit chondrocytes, IL-1 is the only cytokine that can induce metalloprotease genes when added alone, but its activity can be modified by growth factors such as FGF and TGF-β [12–15]. It is thus important to delineate the common pathways that lead to the synthesis of metalloproteases. We have used these complex interactions to demonstrate that the rate of enzyme mRNA induction was significantly higher if FGF was present along with IL-1, than when IL-1 was used alone. We further show that FGF potentiation of IL-1 induced stromelysin but not collagenase levels is likely to be due to the synthesis of new protein(s) within a short time after the addition of IL-1+FGF. These results suggest that collagenase and stromelysin may be subject to different regulatory mechanisms.

The post-receptor signal transduction mechanism in IL-1 or IL-1+FGF-induced cells that lead to the expression of metalloprotease gene is only beginning to be understood. If metalloprotease expression requires co-operativity among several factors, then it is possible that IL-1 may induce low levels of all the critical post-receptor signal transducer(s), whereas FGF could induce one or more, but not all of them. However, a combination of IL-1+FGF could produce higher levels of some or all, leading to greater levels of metalloprotease mRNA. One possible set of candidates that could mediate such effects are the nuclear protooncogenes, c-*fos* and c-*jun*. They are inducible by growth factors and are believed to form dimers that recognize a specific sequence on DNA, namely the AP-1 binding site [1,25–28]. Such sites have been identified in the genes for stromelysin and collagenase [1,25–28]. While we have not addressed this question in this paper, recent studies have shown that in fibroblasts, EGF treatment resulted in the co-induction of stromelysin, c-*fos* and c-*jun* [28]. It is thus possible that while IL-1 induces both c-*jun* and c-*fos*, FGF may induce higher levels of one of the two, facilitating the formation of heterodimers, and ultimately leading to increased transcription of metalloprotease mRNAs.

Interestingly, collagenase induced by either IL-1 or IL-1+FGF was inhibited even when cycloheximide was added 4 h post stimulation. The reason for this difference between collagenase and stromelysin inhibition by cycloheximide is not known, but may reflect subtle differences in regulation of the 2 genes. Although both stromelysin and collagenase were thought to be co-ordinately regulated, recent studies have provided evidence for their independent regulation [23,24]. However, in our studies, this effect was observed only with cycloheximide. TGF-β, another inhibitor of metalloprotease induction, displayed comparable inhibitory effects on collagenase and stromelysin. This effect of TGF-β appeared to be an early event, as it was able to block or reduce mRNA accumulation only if it was added at the same time as IL-1. Thus, the growth factors that positively (FGF) or negatively (TGF-β) modulate the IL-1 activity may do so by affecting early events. An alternate explanation for the distinction between cycloheximide and TGF-β could be that cycloheximide may stabilize the rabbit stromelysin mRNA, but not collagenase mRNA, as reported for the human stromelysin and collagenase genes, respectively, whereas TGF-β may not do so [29]. Thus, complex interactions may regulate the expression of collagenase and stromelysin genes.

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