Primary and passaged human synovial fibroblasts isolated from rheumatoid pannus were treated with recombinant interleukin-1 (IL-1) α or β, tumor necrosis factor-α (TNF), or phorbol myristate acetate (PMA) to determine the effects of these stimuli on the relative expression of stromelysin, collagenase, and tissue inhibitor of metalloproteinases (TIMP). The steady-state mRNA levels for these genes and glyceraldehyde-3-phosphate dehydrogenase were determined on Northern blots. Immunoblot analyses of the conditioned media using monoclonal antibodies generated against recombinant human stromelysin, collagenase, or TIMP showed that protein levels reflected the corresponding steady-state mRNA levels. The results revealed that 1) stromelysin and collagenase were not always coordinately expressed; 2) IL-1 was more potent than TNF or PMA in the induction of stromelysin expression; 3) neither IL-1 nor TNF significantly affected TIMP expression; 4) PMA induced both metalloproteinase and TIMP expression; and 5) the combination of IL-1 plus TNF had a synergistic effect on stromelysin expression. Dose response and time course experiments demonstrated that the synergistic effect of IL-1 plus TNF occurred at saturating concentrations of each cytokine and lasted for 7 days. In summary, the ability of IL-1 and TNF to preferentially induce stromelysin and collagenase expression, versus TIMP, may define a pivotal role for these cytokines in the pathogenesis of rheumatoid arthritis.

To identify the optimum cell culture conditions for performing our studies, both primary and passaged rheumatoid HSF were treated with IL-1 or TNF in the presence or absence of serum. Stromelysin, collagenase, and TIMP expression was examined at both the mRNA and protein levels. The results demonstrated that IL-1 was a potent inducer of metalloproteinase; kb, kilobase; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

**Synergistic Effects of Interleukin-1 and Tumor Necrosis Factor-α on Stromelysin Expression**

(Received for publication, December 20, 1989)

Karen L. MacNaul‡, Nicole Chartrain‡, Michael Larks, Michael J. Tocci‡, and Nancy I. Hutchinson‡‡

*From the ‡Department of Molecular Immunology, ‡Department of Biochemical and Molecular Pathology, Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey 07065*

Rheumatoid arthritis is a chronic inflammatory disease that is characterized by the proliferation of the synovial membrane into a highly vascularized tissue known as pannus, the invasive growth of which corresponds with the progressive destruction of articular cartilage and bone. The pannus consists of several distinct cell types, which include resident synovial fibroblasts and infiltrating mononuclear cells capable of producing a number of inflammatory mediators (1). Previous studies have shown that human synovial fibroblasts (HSF)1 isolated from pannus secrete three neutral metalloproteinases, MMP-1, MMP-2, and MMP-3, upon treatment with monococyte conditioned medium (2). These metalloproteinases correspond to collagenase, gelatinase, and stromelysin and together are capable of degrading the major macromolecular components of cartilage: collagen and proteoglycan (3–5).

Most cells capable of secreting collagenase and stromelysin also secrete a natural tissue inhibitor of metalloproteinases, TIMP (6–9). It has been speculated that the relative expression of TIMP versus collagenase and stromelysin in joint tissues may be critical in altering the balance between the maintenance of articular cartilage and its destruction in diseases such as rheumatoid arthritis. Although several studies have examined the regulation of stromelysin and collagenase in various fibroblasts and have found these genes to be coordinately expressed (10–15); few studies have examined their relative regulation with respect to TIMP (6, 12, 16). More importantly, none of the earlier studies have determined how defined physiologically-relevant cytokines regulate the balanced expression of metalloproteinases versus TIMP in cells isolated from human joint tissues.

In the following studies, we examined the expression of stromelysin, collagenase, and TIMP in rheumatoid HSF treated with purified recombinant human interleukin-1 (IL-1) and tumor necrosis factor-α (TNF). A large body of evidence suggests that these two cytokines may be mediators in the pathogenesis of rheumatoid arthritis. For example, intra-articular injection of IL-1 in rabbits results in proteoglycan loss from articular cartilage (17). IL-1 can induce cartilage degradation and bone resorption (18, 19). In culture, monocyte conditioned medium or IL-1 can induce collagenase and stromelysin expression in synovial fibroblasts (2, 4, 6, 20). Similarly, TNF has many biological activities in common with IL-1 (22, 23).

To identify the optimum cell culture conditions for performing our studies, both primary and passaged rheumatoid HSF were treated with IL-1 or TNF in the presence or absence of serum. Stromelysin, collagenase, and TIMP expression was examined at both the mRNA and protein levels. The results demonstrated that IL-1 was a potent inducer of metalloproteinases.
teinase, but not TIMP, expression. Interestingly, stromelysin and collagenase were not always coordinately expressed. In addition, the combination of IL-1 plus TNF synergistically enhanced stromelysin expression to a greater extent than collageanase expression. Alone or in combination, the ultimate effect of these cytokines was an altered balance of metalloproteinase and TIMP expression in favor of the metalloproteinases. Perhaps, this is one way in which IL-1 and TNF play key roles in the pathogenesis of rheumatoid arthritis.

**MATERIALS AND METHODS**

**Reagents**—All tissue culture reagents were obtained from Gibco. Fully active recombinant human IL-1 α and β were synthesized and purified at Merck Sharp and Dohme Research Laboratories (24). Recombinant human TNF-α was obtained from Dr. Susan H. Socher, Merck Sharp and Dohme Laboratories, West Point, PA. Each of these cytokines was used at a final concentration of 1 nM, unless indicated otherwise. Phorbol myristate acetate (PMA) (Sigma) was dissolved in dimethyl sulfoxide at a concentration of 1 mg/ml and diluted to a final concentration of 100 ng/ml in the appropriate culture medium, unless indicated otherwise. Lactalbumin hydrolysate (LAH) and bovine serum albumin (BSA, essentially globulin-free) were obtained from Worthington Biochemical (Freehold, NJ).

**Culture of Primary HSF**—Primary HSF were isolated from rheumatoid pannus, as described by Chin et al. (25). Cells were grown in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum (FCS), 10 units/ml penicillin, and 100 μg/ml streptomycin. Primary cells were plated 2 x 10^6 cells/25 cm^2 flask, fed every 3 days, and grown in culture for 1 week before use. Passaged cells were split 1:4 and used at passages three and four. For experimentation, the cells were plated into 25-cm^2 flasks, grown to sub-confluence (70-80%), rinsed with serum-free medium, and treated with the appropriate medium with or without stimuli. At the times indicated in the text, the media from the cells were collected, the cells were rinsed with phosphate buffered saline, and total RNAs were extracted. The experiments described in Figs. 2, 3, 4, 6, and 8 were performed with the HSF of a single patient, as were the experiments described in Figs. 5 and 7. Results similar to those described have also been obtained with other rheumatoid arthritis patients (data not shown).

**Total RNA Extraction**—For Northern blot analyses, total RNA was extracted with 4 M guanidine thiocyanate (Fluka Chemical Corp., Switzerland) as described previously (26). Specifically, the RNAs from each 25-cm^2 flask of HSF were extracted with 2 ml of guanidine thiocyanate and precipitated with 0.025 volumes of 1 M acetic acid and 0.75 volumes of 100% ethanol at -20 °C. The precipitate was collected by centrifugation and resuspended in extraction buffer (5% phenol, 5% sodium laurylsarcosine, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA) and extracted with equal volumes of phenol, phenolchloroformisoamyl alcohol (50:48:2), and chloroform:isoamyl alcohol (24:1). The RNA in the final aqueous phase was precipitated by adding 0.1 volumes of 2 M LiCl and 2 volumes of 100% ethanol at -20 °C.

For cDNA cloning, total HSF cell lysates extracted with 4 M guanidine thiocyanate were layered over a 6 M CsCl cushion and centrifuged at 25,000 rpm for 24 h. The pellet was resuspended in extraction buffer and extracted with phenol:chloroform:isoamyl alcohol as described above. The RNA was precipitated with 1 volumes 2 M LiCl, and 2 volumes of 100% ethanol at -20 °C.

**Cloning and Characterization of Human Stromelysin, Collagenase, and TIMP cDNAs**—Primary HSF, isolated from human pannus, were treated with 1 nM recombinant IL-1 β for 24 h. Total RNA was isolated as described above. The polyadenylated mRNA was selected by oligo(dT) chromatography (27). Approximately 480 ng of double-stranded cDNA was synthesized by the method of Gubler and Hoffman (28) with the Amersham Corp. cDNA kit. The double-stranded cDNA was C-tailed with terminal deoxynucleotidyltransferase and annealed with G-tailed pBR322 DNA (Bethesda Research Laboratories) as described previously (29). The annealed DNAs were transformed into Escherichia coli strain MC1061 (30). Eighty-six thousand individual clones were generated.

The cDNA library was screened by colony hybridization (31) with two 20-mer oligonucleotides synthesized to the 5’ coding regions of each of the three mRNAs (the sequence of the human stromelysin cDNA was kindly provided by G. Murphy, Strangways Research Laboratories, Cambridge, Great Britain). Individual clones were isolated, grown, and plasmid DNAs isolated by the method of Birnboim and Doly (32). The size and identity of each cDNA clone was confirmed by restriction map and Southern blot analyses (data not shown). Full-length human cDNA clones for stromelysin, collagenase, and TIMP were isolated.

**Northern Blot Detection of Stromelysin, Collagenase, and TIMP mRNA**—The steady-state levels of each mRNA (i.e. the levels resulting from the cumulative effects of both mRNA transcription and mRNA degradation) were determined on Northern blots. Equivalent amounts of total RNA from each sample (-3 μg) were electrophoresed on 1% agarose, 2.3 M formaldehyde gels (27). The gels were stained with ethidium bromide before transfer to check RNA integrity and to confirm the amounts of each RNA sample loaded (data not shown). The RNAs were blotted onto nitrocellulose in 20 x SSC (3 M sodium chloride, 0.3 M sodium citrate (pH 7.0)). The blots were baked at 80 °C under vacuum and hybridized with ^32P-labeled nick-translated cDNA inserts for either stromelysin, collagenase, TIMP (cloned as described above), or glyceraldehyde-3-phosphate dehydrogenase (kindly provided by Dr. Robert W. Allen, American Red Cross Blood Service, St. Louis, MO).Nick translations were performed with the Amersham Corp. nick-translation kit. Each probe had a specific activity between 1.8-2.1 x 10^6 cpm/μg. The stromelysin probe was a 1.8-kb Psul cDNA fragment. A 1.5-kb Xbol cDNA fragment was used as the collagenase probe. The TIMP probe was a 0.9-kb Hincll fragment that contained 0.3 kb of pBR322 sequences. A 1.0-kb EcoRl fragment of the 1.5-kb insert was used as the glyceraldehyde-3-phosphate dehydrogenase probe. The hybridizations were performed as described previously (33). The filters were exposed to Kodak X-Omat film with a Du Pont lightening intensifying screen at -80 °C. The stromelysin, collagenase, TIMP, and glyceraldehyde-3-phosphate dehydrogenase cDNAs did not cross-hybridize under these conditions (data not shown).

**Immunoblot Detection of Stromelysin, Collagenase, and TIMP Proteins**—One monoclonal antibody to human prostromelysin, two to procollagenase, and two to TIMP were purchased from Cellect Ltd., Berkshire, Great Britain. Purified recombinant stromelysin and TNF were obtained from purified recombinant collagenase were used to demonstrate the specificity of the antibodies (data not shown). Aliquots of HSF conditioned media were brought to a final volume of 100 μl with Dulbecco’s modified Eagle’s medium plus 0.2% BSA, centrifuged to remove cellular debris, mixed with 10 μl of 1% Chaps (in Tris-buffered saline, 10 mM Tris-HCl (pH 7.5), 250 mM NaCl and 100 μl of 2 x TMB (1 X = 10 mM Tris-HCl (pH 7.5), 250 mM NaCl, 20% methanol). Each sample was then applied, in triplicate, to TMB-equilibrated nitrocellulose filters mounted in a slot blot apparatus. The wells of the apparatus were washed with 500 μl of 0.05% Tween 20, at 37 °C, overnight.

The nitrocellulose filters were prepared for immuno-detection of stromelysin, collagenase, or TIMP by blocking with M Blotto (10%) w/v Carnation non-fat dry milk, 10 mM Tris-HCl (pH 7.5), 0.9% NaCl for 2 h at 37 °C, with constant shaking. The filters were then treated with either anti-stromelysin, anti-collagenase, or anti-TIMP monoclonal antibodies, diluted to 2.5 μg of each IgG/ml of M-Blotto, overnight at room temperature.

Biotin-labeled anti-mouse IgG secondary antibodies and ^125l-labeled streptavidin (Amersham Corp.) were diluted 1:200 in M-Blotto and used to detect the antigen-antibody complexes. Exensive washes in 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 3 mM EDTA, 0.05% Tween 20, at 37 °C were performed between each step. Autoradiography results generated from Northern blot or immunoblot analyses were quantified using an LKB Densitometer. Multiple autoradiographic exposures were examined to insure that the results analyzed reflected those produced in the linear range of the film. For calculating, specific inductions of stromelysin, collagenase, and TIMP mRNAs under different conditions, the densitometric values were standardized to a constant level of glyceraldehyde-3-phosphate dehydrogenase mRNA levels in the corresponding induced and uninduced samples (treated with 10% FCS or 0.2% BSA). Glyceraldehyde-3-phosphate dehydrogenase mRNA is constitutively expressed and can be used as a standard for nonspecific mRNA inductions or variations in RNA quantities loaded onto gels. Comparisons of stromelysin, collagenase, and TIMP mRNA expression levels confirmed that with similar experimental conditions (i.e. in the presence or absence of serum) glyceraldehyde-3-phosphate dehydrogenase mRNA levels reflected total RNA levels (data not shown). Therefore, the relative inductions of each mRNA were calculated, first, by dividing the densitometer...
RESULTS

Analysis of Stromelysin, Collagenase, and TIMP mRNA Expression in Primary and Passaged Rheumatoid HSF—To determine the optimum conditions for comparing the effects of IL-1 and TNF on stromelysin, collagenase, and TIMP expression in rheumatoid HSF, both primary and passaged HSF cultures were treated with saturating concentrations of IL-1 \( \beta \), TNF-\( \alpha \), PMA, or IL-1 \( \alpha \) (passaged HSF only) in the presence or absence of FCS. One nanomolar IL-1 or TNF was considered saturating, since the \( K_d \) values for their receptors have been reported to be in the picomolar range (25, 34). To prevent protein deprivation of the cells under serum-free conditions, the serum-free medium was supplemented with 0.2% BSA. In preliminary experiments, we found that supplementing the medium with some preparations of 0.2% LAH resulted in the induction of stromelysin and collagenase mRNA expression without the addition of other stimuli. Therefore, LAH was not used as a protein supplement for these experiments; but an LAH-treated control was included for comparison.

Total RNA was isolated from the primary and passaged HSF 24 and 72 h after treatment with stimuli. The levels of stromelysin, collagenase, and TIMP mRNA were determined by Northern blot analyses. In addition, the levels of the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase mRNA were determined.

The primary HSF constitutively expressed the mRNAs for stromelysin, collagenase, TIMP, and glyceraldehyde-3-phosphate dehydrogenase (Fig. 1). By 72 h, every sample had similar patterns of mRNA expression. Thus, the addition of IL-1, TNF, or PMA did not significantly alter the balance of metalloproteinase versus TIMP expression in this primary HSF culture.

In the passaged HSF cultures (Fig. 2), the pattern of mRNA expression differed dramatically from that seen in the primary cultures. Passaged HSF grown in 10% FCS or 0.2% BSA (lanes 1 and 3, 24 and 72 h, respectively) did not express detectable levels of stromelysin or collagenase mRNAs at 24 or 72 h. However, TIMP and glyceraldehyde-3-phosphate dehydrogenase mRNA were detected. The addition of IL-1, TNF, PMA, or LAH to the medium induced the expression of stromelysin and collagenase mRNA; yet, the pattern of expression was different for each of the stimuli tested (lanes 2 and 4–10). The ability of exogenously added stimuli to differentially affect the expression of stromelysin, collagenase and TIMP mRNA in passaged HSF indicated that these cultures were the most appropriate for further studies on the regulation of these genes.

Quantification of Stromelysin, Collagenase, and TIMP mRNA Levels Induced by IL-1, TNF, or PMA—To quantify the effects of IL-1, TNF, or PMA on the relative expression of stromelysin, collagenase, and TIMP mRNA in passaged HSF cultures, the autoradiograms shown in Fig. 2 were scanned by densitometer. The specific inductions were calculated and standardized with respect to corresponding glyceraldehyde-3-phosphate dehydrogenase mRNA levels (Fig. 3). For stromelysin and collagenase, this number indicates the minimum induction, since there was no detectable expression of either of these mRNAs in the uninduced cultures (lanes 1 and 3, 24 and 72 h, respectively).

Steady-state levels of stromelysin mRNA were highest in the presence of IL-1, with a 30-fold induction under serum-free conditions. IL-1 \( \alpha \) and \( \beta \) were equally potent in inducing stromelysin mRNA expression. TNF and PMA induced stromelysin mRNA only 2- to 3-fold under similar conditions. In contrast, each of these stimuli were similar in their abilities to induce collagenase mRNA expression. The differential effects of IL-1 and TNF on stromelysin versus collagenase mRNA expression indicated that these two genes were not always coordinately expressed in rheumatoid HSF. By 72 h, IL-1-induced stromelysin mRNA expression to significantly higher levels than collagenase, whereas TNF induced predominantly collagenase mRNA expression. With either stimuli, stromelysin and collagenase mRNA levels were greatest under serum-free conditions. Therefore, all subsequent experiments were performed in the absence of serum (0.2% BSA). TIMP mRNA expression was virtually unaffected by either IL-1 or...
Stromelysin, Collagenase, and TIMP Expression in HSF

Figure 3. Differential effects of IL-1, TNF, and PMA on stromelysin, collagenase, and TIMP mRNA expression. The relative inductions of stromelysin (upper panel), collagenase (middle panel), and TIMP (lower panel) steady-state mRNA in passaged rheumatoid HSF treated with IL-1α, IL-1β, TNF-α, or PMA in the presence of 10% FCS (F) or 0.2% BSA (B) for 24 (closed bars) or 72 (open bars) h. All calculations of relative induction were equalized with respect to glyceraldehyde-3-phosphate dehydrogenase mRNA expression.

Figure 4. Comparison of primary and passaged cell morphologies. Photographs of primary (A and B) and passaged (C–F) rheumatoid HSF untreated (A and C) or treated with IL-1β (B and D), TNF (E), or PMA (F).

In contrast, passaged HSF cultures, when left untreated (Fig. 4C) appeared flatter and more fibroblast-like. The addition of IL-1 induced the appearance of highly rounded dendritic-shaped cells (D). The ability of murine IL-1 to transform passaged rheumatoid HSF into "dendritic" cells has been reported previously (36). TNF-treated HSF were flat and stringy in appearance (E), while PMA caused the cells to pull away from each other and take on an elongated morphology. Thus, in both primary HSF and IL-1-induced passaged HSF, the highly rounded dendritic morphology corresponded with the highest levels of stromelysin mRNA expression and the highest ratio of metalloproteinase to TIMP mRNA, but did not correspond with collagenase mRNA levels.

Dose-Response Effects of IL-1, TNF, and PMA on Metalloproteinase and TIMP Expression—The minimum concentrations of IL-1, TNF, and PMA required to stimulate stromelysin and collagenase mRNA expression were determined in a dose-response experiment, to compare the relative potencies of these stimuli. Passaged HSF were treated with concentrations of IL-1β or TNF ranging from 0.1 pM to 1 nM or PMA from 0.1 ng/ml to 1 μg/ml. Total RNA was extracted 24 and 72 h after the addition of stimuli and assayed for stromelysin, collagenase, TIMP, and glyceraldehyde-3-phosphate dehydrogenase mRNAs on Northern blots.

The results of the 72-h time point are shown in Fig. 5. IL-1 was able to induce maximal stromelysin and collagenase mRNA expression at a concentration as low as 1 pM, and metalloproteinase mRNA levels were maintained over a 1000-fold concentration range (1 pM to 1 nM). TNF did not induce maximal levels of stromelysin and collagenase mRNA expression until approximately 50 pM. Therefore, TNF did not appear to be as potent an inducer of stromelysin and collagenase mRNA expression as IL-1. PMA induced maximal stromelysin, collagenase, and TIMP mRNA expression at 10 ng/ml, and maximal mRNA levels were observed at 24 h (data not shown). These results confirmed that passaged HSF under serum-free conditions provided a sensitive culture system for studying the roles of specific cytokines on the regulation of stromelysin, collagenase, and TIMP mRNAs in rheumatoid HSF and that the steady-state levels of these mRNAs were differentially expressed in the presence of IL-1 and TNF.

IL-1 Plus TNF Synergistically Enhance Stromelysin mRNA Expression—The combined effects of IL-1 plus TNF on stromelysin, collagenase, and TIMP expression in rheumatoid HSF was determined in a mixing experiment. Passaged HSF were treated with either IL-1 (α or β), TNF, or PMA, alone or in combination. In some cases, indomethacin was added to determine whether the effects of these cytokines were mediated by prostaglandins. The conditioned medium and total RNA from these cells were collected 24 and 72 h after treatment. The RNA was analyzed on Northern blots and the...
results of the 72 h samples are shown in Fig. 6.

The results are similar to those described in Figs. 2 and 3. IL-1 (α or β) induced stromelysin mRNA expression to much higher levels than TNF or PMA (lanes 1–5). As expected, saturating amounts of both IL-1 α plus IL-1 β did not have an additive effect on the induction of stromelysin or collagenase mRNA expression (lane 6). Since both IL-1 molecules bind to the same receptor on fibroblasts (25), they presumably affect metalloproteinase mRNA expression via the same mechanism. Again, TNF induced collagenase mRNA expression to a greater extent than stromelysin mRNA (lane 4). However, an unexpected result was the effect that the combination of IL-1 plus TNF had on stromelysin mRNA expression. In combination, IL-1 plus TNF substantially enhanced the induction of stromelysin mRNA (lane 7), to a level 3-fold higher than would be expected from a simple additive effect of both cytokines (densitometry not shown). Yet, the combination of IL-1 plus TNF appeared to have a less dramatic effect on collagenase mRNA expression and no significant effect on TIMP mRNA expression.

In addition, indomethacin did not inhibit the induction of stromelysin or collagenase mRNA by IL-1 or TNF. In contrast, indomethacin plus IL-1 or TNF appeared to slightly enhance metalloproteinase mRNA expression (lanes 9 and 10); whereas, indomethacin alone appeared to nonspecifically depress mRNA expression (lane 11). These results indicated that prostaglandins did not mediate the induction of stromelysin or collagenase mRNA expression by IL-1 or TNF.

Long-term Synergistic Effects of IL-1 Plus TNF on Stromelysin mRNA Induction—To determine whether the synergistic effect of IL-1 plus TNF on stromelysin mRNA expression was a short- or long-term effect, a time course experiment was performed. Passaged HSF were treated with IL-1, TNF, PMA, or IL-1 plus TNF, for various times between 16 h and 7 days. Total RNA was extracted at the times indicated and analyzed on Northern blots (Fig. 7A). Quantitative analyses of these results were determined by densitometry (Fig. 7B). A dramatic synergistic induction of stromelysin mRNA expression was evident in the presence of IL-1 plus TNF and was maintained to the 7-day time point. In the same samples, collagenase mRNA expression was not enhanced to a similar extent. TIMP mRNA levels were most highly induced by PMA. In general, PMA induced maximal stromelysin, collagenase, and TIMP mRNA levels, whereas, indomethacin alone appeared to nonspecifically depress mRNA expression (lanes 9 and 10); whereas, indomethacin alone appeared to nonspecifically depress mRNA expression (lane 11). These results indicated that prostaglandins did not mediate the induction of stromelysin or collagenase mRNA expression by IL-1 or TNF.

FIG. 5. Dose-response experiment examining the effects of IL-1, TNF, and PMA on stromelysin, collagenase, and TIMP mRNA expression. Northern blot analyses of the total RNA isolated from passaged rheumatoid HSF treated with IL-1 β, TNF-α, or PMA at the concentrations indicated. The Northern blots were hybridized as described in Fig. 1. A shows the autoradiographs of the Northern blots. B depicts the densitometer analyses of these autoradiographs equalized with respect to glyceraldehyde-3-phosphate dehydrogenase mRNA expression. Closed circles, closed squares, and closed triangles represent stromelysin, collagenase, and TIMP mRNAs, respectively. The vertical arrows indicate the concentrations at which each treatment induced maximal mRNA expression.

FIG. 6. Synergistic induction of stromelysin mRNA expression in passaged HSF treated with IL-1 plus TNF. Northern blot analysis of total RNA isolated from passaged rheumatoid HSF, untreated (lane 1) or treated with IL-1 β (lane 2), IL-1 α (lane 3), TNF-α (lane 4), PMA (lane 5), IL-1 β plus IL-1 α (lane 6), IL-1 β plus TNF-α (lane 7), IL-1 β plus TNF-α plus PMA (lane 8), IL-1 β plus indomethacin (lane 9), TNF-α plus indomethacin (lane 10), indomethacin alone (lane 11), or triple the standard concentration of IL-1 β (lane 12) for 72 h. The Northern blots were hybridized as described in Fig. 1.

Determination of Stromelysin, Collagenase, and TIMP Protein Levels by Immunoblot Analysis—To correlate protein synthesis with steady-state mRNA levels, the 72-h conditioned media from the HSF described in Fig. 6 were assayed on immunoblots (Fig. 8A). The primary antibodies were monoclonals generated against purified recombinant human prostromelysin, procollagenase, or TIMP. The results were quantified by densitometer analysis and depicted as the rela-
examining the effects of medium alone (lane blots were hybridized as described in Fig. 1. B, densitometer analysis PMA, IL-l, TNF, or IL-1 plus TNF. A, Northern blot analysis examining the effects of medium alone (lane A) or medium plus either PMA (lane B), IL-1 (lane C), TNF (lane D), or IL-1 plus TNF (lane E) on passaged rheumatoid HSF for 16 h to 7 days. The Northern blots were hybridized as described in Fig. 1. B, densitometer analysis of the Northern blots. The results are graphed as the relative area of each mRNA band with respect to time (hours) and various treatments: open triangles depict PMA; open squares depict TNF; closed squares depict IL-1; and closed circles depict IL-1 plus TNF. Stromelysin mRNA levels remained elevated throughout the 7-day time point.

Densitometric analysis of each protein with respect to the uninduced sample (0.2% BSA, Fig. 8B). Stromelysin was induced 30- to 50-fold by IL-1 (α, β, or α plus β), but only 2- to 3-fold by TNF or PMA. In the presence of IL-1 plus TNF, stromelysin was induced approximately 175-fold. This is almost 4-fold greater than would be expected from an additive effect of both cytokines. The combination of IL-1, TNF, plus PMA resulted in an even greater (300-fold) induction of stromelysin. Collagenase was induced 8- to 10-fold with either IL-1 (α, β, or β plus β) or TNF alone. The combination of IL-1 plus TNF resulted in a 14-fold induction, which indicated only an additive effect of these two cytokines. The addition of PMA to IL-1 plus TNF resulted in a slightly higher level of induction: 24-fold. TIMP levels were similar under all of the conditions tested. This result reflects the small transient nonspecific nature of TIMP induction by PMA and the relative insignificance of this induction compared with the constitutive expression of TIMP. In conclusion, the relative effects of IL-1, TNF, and PMA on stromelysin, collagenase, and TIMP steady-state mRNA levels were reflected in the relative levels of each protein synthesized and secreted into conditioned medium.

**DISCUSSION**

This report characterized the abilities of the monokines IL-1 and TNF to alter the relative expression of stromelysin and collagenase versus their natural inhibitor, TIMP, in HSF isolated from rheumatoid pannus. Initially, the effects of these two monokines on the steady-state expression of the mRNAs for each gene were examined in both primary and passaged HSF, in the presence or absence of serum, to identify the optimum in vitro culture conditions for performing these studies.

Primary rheumatoid HSF constitutively expressed high levels of stromelysin mRNA, followed by collagenase and TIMP mRNAs. The absolute levels of stromelysin and collagenase mRNAs constitutively expressed in uninduced primary cultures varied from patient to patient (data not shown), which may reflect differences in the heterogeneous population of cells plated in individual primary cultures. However, in the experiment described in Fig. 1, metalloproteinase mRNA expression could not be induced further by the addition of IL-1 or TNF. In the presence of 10% FCS, slightly lower levels of stromelysin and collagenase mRNA were observed, but these levels increased by 72 h.

In contrast, passaged HSF did not constitutively express high levels of stromelysin and collagenase mRNAs at either 24 or 72 h, but the addition of IL-1 induced a pattern of stromelysin, collagenase, and TIMP mRNA expression similar to that observed in primary HSF cultures. The lower level of stromelysin and collagenase mRNA expression observed in the presence of 10% FCS suggested the possibility that FCS contained one or more factors that negatively affected the steady-state expression of these mRNAs or partially blocked the activities of the added stimuli.

The constitutive expression of metalloproteinase mRNA in primary HSF cultures and the ability of IL-1 to induce a similar pattern of mRNA expression in passaged HSF indicated that the primary HSF cultures were self-induced. It has been shown that primary rheumatoid HSF cultures contain a significant subpopulation of macrophages (35). In situ hybridization and immunofluorescence experiments have demon-
strated that these macrophages express IL-1 (37).2 Passing rheumatoid HSF results in the loss of macrophages (36) and an endogenous source of cytokines.

In the absence of serum, the medium was supplemented with either 0.2% BSA or LAH. LAH was tested as a protein supplement, since it had been reported that LAH did not induce stromelysin mRNA expression in normal rabbit synovial fibroblasts (10, 11). Saus et al. (4) also used LAH-containing medium for their studies examining the effect of IL-1 on stromelysin and collagenase mRNA expression in rheumatoid HSF. However, in the latter study, the effect of LAH alone was not shown. In our studies (Fig. 5), passaged rheumatoid HSF switched to medium containing 0.2% LAH expressed high levels of stromelysin and collagenase mRNAs in the absence of any other stimuli. A similar induction was also observed with human gingival fibroblasts (data not shown). How metalloproteinase mRNA expression was induced in the presence of LAH and whether the induction was specific for human fibroblasts or fibroblasts isolated from inflamed tissues is under investigation. BSA did not induce stromelysin and collagenase mRNA expression in rheumatoid HSF, nor did it induce metalloproteinase mRNA expression when mixed with LAH (data not shown). Therefore, the remainder of our studies examined the effects of exogenously added stimuli on the expression of stromelysin, collagenase, and TIMP in passaged rheumatoid HSF grown in serum-free medium supplemented with 0.2% BSA.

The addition of purified recombinant IL-1 or TNF to passaged HSF could differentially induce stromelysin and collagenase steady-state mRNAs levels, while not inducing TIMP mRNA levels. TIMP mRNA expression could be induced by the presence of PMA. In Fig. 3, stromelysin mRNA levels were induced 30-fold by IL-1 or β but not induced significantly by TNF, whereas collagenase mRNA expression was induced similarly by either IL-1 or TNF. This provided one indication that stromelysin and collagenase mRNAs were not always coordinately expressed in rheumatoid HSF. However, the degree to which TNF induced stromelysin mRNA expression in HSF varied among patients. For example, in Fig. 5, HSF from another patient were used, and IL-1 and TNF were similar in their abilities to induce stromelysin mRNA expression. We have performed similar induction experiments using HSF from additional patients and, again, have observed responses similar to those of either Fig. 3 or 5 (data not shown).

Further evidence for the coordinate expression of stromelysin and collagenase was obtained from the synergy and time course experiments. First, IL-1 plus TNF synergistically enhanced stromelysin mRNA expression, resulting in stromelysin mRNA levels 3- to 4-fold greater than would be expected from a simple additive effect of both cytokines; yet IL-1 plus TNF appeared to have had only an additive effect on collagenase mRNA expression. Second, the time course experiment demonstrated high levels of stromelysin mRNA at times when collagenase mRNA levels had already declined. The ability of HSF to express high levels of stromelysin mRNA under conditions when collagenase mRNA is relatively low may be relevant in light of recent data demonstrating that stromelysin levels in rheumatoid synovial fluids are an average of 10-fold higher than collagenase levels, as determined by ELISA.2

The demonstration of coordinate expression of stromelysin, collagenase, and TIMP in the studies presented here contrast several earlier studies. However, previous studies describing coordinate expression of collagenase and stromelysin, or TIMP (2, 6, 10–15) differed from ours in that either the cells examined were not human synovial fibroblasts, or the stimuli used were nonphysiologic (PMA) or ill-defined (monocyte conditioned medium). In several studies, the inductions observed were low, and the results were not standardized with respect to a constitutively expressed gene, such as glyceraldehyde-3-phosphate dehydrogenase.

The time course experiment (Fig. 7) also demonstrated that maximal IL-1 or TNF induction of stromelysin and collagenase mRNA expression was observed relatively late, with mRNA levels peaking approximately 24–72 h after induction. This observation is consistent with the hypothesis that IL-1-mediated induction of metalloproteinase expression involves the expression of an intermediate gene or second messenger. This intermediate did not appear to involve the synthesis of prostaglandins, since indomethacin did not inhibit IL-1 induction of stromelysin or collagenase expression; in fact the addition of indomethacin appeared to enhance metalloproteinase expression. The inability of indomethacin to inhibit collagenase induction has also been reported by Dayer et al. (35). The requirement for an intermediate gene product in the IL-1 induction of metalloproteinase expression is also supported by studies demonstrating that protein synthesis inhibitors can prevent the IL-1 induction of collagenase (39) and stromelysin (data not shown) mRNA expression. In addition, the late time of maximal metalloproteinase mRNA expression indicates that the optimal time to examine transcriptional effects of IL-1 may be later than has been examined in some earlier studies (21).

To what extent cytokine induction of increased stromelysin steady-state mRNA levels was mediated through increased transcription or increased mRNA half-life is not known. However, the ability of stromelysin steady-state mRNA levels to remain high for 1 week, while collagenase mRNA levels decreased between 4 and 7 days, suggests that either the half-life of stromelysin mRNA was greater than collagenase mRNA or the stromelysin gene remained transcriptionally active longer.

Finally, the observation that stromelysin, collagenase, and TIMP mRNA levels reflected the subsequent accumulation of each protein in the conditioned medium indicates that the regulation of these genes occurred primarily at the mRNA level. Therefore, in the presence of IL-1 or TNF the balance of metalloproteinase versus TIMP expression in rheumatoid HSF is altered in favor of the metalloproteinases at both the mRNA and protein levels.

In summary, the ability to specifically induce stromelysin and collagenase expression in rheumatoid HSF, without inducing TIMP, may define a pivotal role for IL-1 and TNF in the pathogenesis of rheumatoid arthritis and cartilage destruction. In the future, we would like to examine the expression of other members of the neutral metalloproteinase gene family (40, 41) and TIMP-2, the recently identified second member of the TIMP gene family (38, 42). However, a key question to be addressed is how these genes are expressed in vivo in normal and diseased joint tissues.

Acknowledgments—We would like to thank Jerry DeSalvo for his assistance in obtaining densitometry analyses of our Northern and immuno-blot results. We would also like to thank Drs. Gideon Rodan and Philip Davies for their critical reading of the manuscript.

REFERENCES


M. Lark, manuscript in preparation.
Stromelysin, Collagenase, and TIMP Expression in HSF 17245

Discoordinate expression of stromelysin, collagenase, and tissue inhibitor of metalloproteinases-1 in rheumatoid human synovial fibroblasts. Synergistic effects of interleukin-1 and tumor necrosis factor-alpha on stromelysin expression.

K L MacNaul, N Chartrain, M Lark, M J Tocci and N I Hutchinson


Access the most updated version of this article at http://www.jbc.org/content/265/28/17238

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/28/17238.full.html#ref-list-1