Stimulation of procollagenase synthesis in human rheumatoid synovial fibroblasts by mononuclear cell factor/interleukin 1

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Received 17 August 1985

In order to define mechanisms regulating the synthesis of procollagenase in human rheumatoid synovial fibroblasts, the proteins synthesized by cultured cells were labeled with [^{35}S]methionine. Labeled medium proteins were analyzed by SDS-PAGE directly and after immunocomplexing with a specific antibody to human fibroblast collagenase. Labeling of both the predominant form of the enzyme ($M_r \sim 55\,000$) as well as a minor species ($M_r \sim 61\,000$) was increased following incubation with the monokine, mononuclear cell factor/interleukin 1. The ~61 kDa form of the procollagenase appears to be a glycosylated form of the ~55 kDa precursor based on binding to Con A-Sepharose and decrease in the ~61 kDa form after culture in the presence of tunicamycin. Thus, mononuclear cell factor, homologous with interleukin 1, partially purified from monocyte conditioned medium increases incorporation of [^{35}S]methionine into several medium proteins, including those complexed by the anticollagenase antibody. In the presence of mononuclear cell factor/interleukin 1, labeling of the procollagenase was increased 12–14-fold over control cultures incubated with medium alone. Therefore, one of the mechanisms involved in increase of collagenase activity in the medium of cultured synovial fibroblasts in the presence of mononuclear cell factor/interleukin 1 is a stimulation of enzyme protein synthesis.

Procollagenase *Synovial fibroblast* *Mononuclear cell factor* *Interleukin 1*

1. INTRODUCTION

Animal collagenases initiate the degradation of collagen by a specific and limited cleavage across the 3 chains that comprise the collagen molecules in solution or in fibrils. These enzymes appear to play a role in the turnover of several connective tissues [1–3]. Collagenolysis is regulated at several levels including transcription of procollagenase genes, packaging and secretion of newly synthesized procollagenase, activation of the procollagenase and regulation of the activity of the active enzyme by specific inhibitors [3].

We have demonstrated that rheumatoid synovial fibroblasts are the major source of procollagenase [4,5]. When these cells are cultured the levels of procollagenase, measured after activation with trypsin, decrease but can be restored by incubation of the cultured cells with a soluble factor, termed mononuclear cell factor (MCF), secreted by monocyte/macrophages [5–7]. This factor which has been partially purified [8] is probably identical to interleukin 1 (IL1) [9,10].

The present studies were designed to determine whether the increased level of collagenase activity in medium conditioned by the rheumatoid synovial cells stimulated with MCF is accounted for by increased synthesis of enzyme, increased secretion of stored enzyme, or modification of enzyme to increase its enzyme activity. By measuring incorporation of [^{35}S]methionine into secreted proteins as well as those immunocomplexed with a specific antibody to collagenase, we have obtained evidence that MCF stimulates synthesis of procollagenase in synovial target cells.

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Abbreviations: Con A, concanavalin A; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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2. MATERIALS AND METHODS

2.1. *Cell culture and preparation of MCF*

Cultures of human rheumatoid synovial fibroblasts were prepared from samples of synovium obtained at time of surgery from patients with rheumatoid arthritis as described [4,11]. Cells in the first or second passage at a density of 1×10^6 cells per 10 cm dish cultured in Dulbecco's modification of Eagle's medium (DME medium, Gibco) containing 10% fetal calf serum, were exposed to MCF for 48 h prior to the addition of [^{35}S]methionine. Following removal of the serum-containing DME medium, [^{35}S]methionine was added in methionine free-DME medium at 25–50 μCi/ml in a total volume of 5 ml per 100 cm diameter culture dish. Labeled medium was aspirated following 24 h of incubation, unless noted otherwise, then centrifuged at 3000 rpm for 15 min and the supernatant stored at −20°C.

MCF was prepared from peripheral blood monocytes and partially purified on columns of Ultrogel AcA54 as in [8]. Only mononuclear cells adhering to plastic culture dishes after 1 h incubation and extensive washing were utilized. The amount of MCF utilized here was arbitrarily chosen on the basis of stimulation of collagenase by rheumatoid synovial fibroblasts, measured after activation with trypsin [11,12].

2.2. *Preparation of anticollagenase antibody*

Rabbit antibody to human skin fibroblast procollagenase [13,14] was generously provided by Dr Eugene Bauer, Washington University, St. Louis, MO. The immunoglobulin fraction was purified by passing 1 ml of the antibody-containing rabbit serum diluted 1:1 with phosphate-buffered saline (PBS) over a column of protein A-Sepharose CL-4B (Sigma, St. Louis, MO) and eluting the immunoglobulin with 0.1 M glycine, pH 3.0. The immunoglobulin fraction was dialyzed against PBS, containing 0.02% sodium azide, and concentrated to 125 μg/ml in an Amicon apparatus using a UM-10 filter (Amicon, Lexington, MA).

2.3. *Immunocomplexing of procollagenase*

After labeling of synovial cell cultures with [^{35}S]methionine, cell free-medium (100–200 μl) was incubated with 300 μl PBS containing 0.1% Triton X-100, 0.1% bovine serum albumin and antibody, 125 μg/ml, for 1 h at 37°C and an additional 12–14 h at 4°C. Immune complexes were harvested by the addition of 75 μl of a 10% suspension of inactivated *Staphylococcus aureus* cells (Cowan I strain) as a source of immobilized protein A (IgGSORB, The Enzyme Center, Boston, MA) in incubation buffer. The IgGSORB immunocomplexes were washed 3 times with incubation buffer, before the addition of SDS-PAGE sample buffer containing 5% β-mercaptoethanol. The samples were then heated to 90°C for 3 min and centrifuged prior to SDS-PAGE.

2.4. *Binding of proteins to Con A-Sepharose*

Columns were prepared using siliconized glass tubes (0.5 cm diameter). In most experiments 1.5 ml Con A-Sepharose (Pharmacia, Piscataway, NJ) was added, and columns equilibrated with PBS. The labeled sample (0.25 ml) was added followed by PBS and the unbound fraction, usually in a volume of 2.5 ml, collected. Following washing with 6 × the bed volume the bound fraction was eluted with ~2 ml of 0.5 M α-methylmannoside. These samples were then analyzed by SDS-PAGE with or without prior complexing with antihuman collagenase immunoglobulin as described above.

2.5. *Electrophoretic analysis of labeled proteins*

Standard discontinuous SDS-PAGE was performed [15] either with 12.5% polyacrylamide gels or 10–15% linear gradient gels, run at 12–16 mA for 16–18 h at room temperature. Fluorograms of the gels were prepared as described [16]. To quantitate radioactivity, in some experiments bands corresponding to the radioautographs were cut from the dried slab gels extracted with NCS tissue solubilizer (Amersham/Searle, Arlington Heights, IL) and counted using a Packard Tricarb liquid scintillation counter.

2.6. *Measurement of collagenase activity*

Procollagenase was activated with 5–25 μg TPCK trypsin (Worthington, Freehold, NJ) per 100 μl cultured medium for 15 min at 20°C. A 3-fold excess of soybean trypsin inhibitor (Worthington) was then added to stop the reaction [4,11]. Viscometric assays [17] were performed with 1 ml capacity viscometers with flow times for

water of 33 s at 27°C. A volume of the activated sample (100 μl) was then added to a reaction mixture containing 200 μl rat tail tendon collagen (3 mg/ml) and 700 μl of 0.1 M Tris-HCl, pH 7.6, containing 0.15 M NaCl and 0.005 M $CaCl_2$. Purified immunoglobulin (125 μg) was added to the reaction mixture as indicated.

2.7. *Reagents*

[^{35}S]Methionine (spec. act. 1200–1400 Ci/mmol) was purchased from Amersham/Searle. Tunicamycin and α-methyl-D-mannoside were purchased from Sigma.

3. RESULTS

Synovial fibroblasts incubated with the MCF preparation used in the labeling experiments increased the release of latent collagenase activity into medium as shown in the viscometric assay described in fig.1. A viscometric assay was used because the relatively large amount of radioactivity present in medium from cells incubated with [^{35}S]methionine would obscure measurement of solubilization of radioactively labeled collagen fibrils utilized for most collagenase assays. The change in the slope of specific viscosity (η_{sp}) as a function of time was increased ~10-fold in medium from cells incubated with MCF compared to cells incubated with DME medium alone. The specificity of the antibody for collagenase was confirmed by the complete inhibition of collagenase activity by the addition of the anticollagenase antibody as measured by no further decrease in η_{sp}.

The effect of pre-incubation with MCF on the synthesis of labeled proteins by synovial fibroblasts is shown in fig.2A. An increase in the synthesis of several different proteins was observed. The stimulation appeared to be selective, since the labeling of all proteins was not increased to the same degree. The results of immunocomplexing of labeled medium with the antibody to

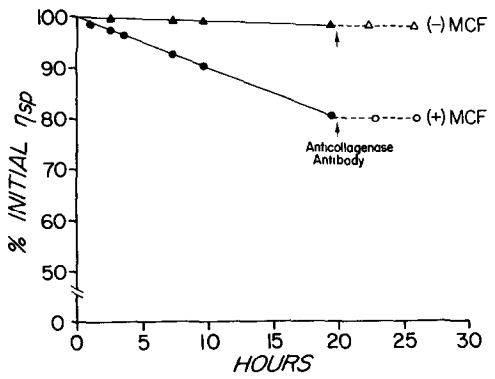

Fig.1. Effect of incubation with MCF on collagenase activity released by synovial fibroblasts. Synovial fibroblasts in second passage were incubated with (+) or without (−) MCF. An aliquot of the cell-free culture medium (100 μl) was then activated with TPCK trypsin as described. The activated medium was then added to a solution of rat tail tendon collagen and specific viscosity (η_{sp}) determined at the intervals indicated. At the time indicated by the arrows the purified immunoglobulin fraction (125 μg) of the rabbit antihuman collagenase was added.

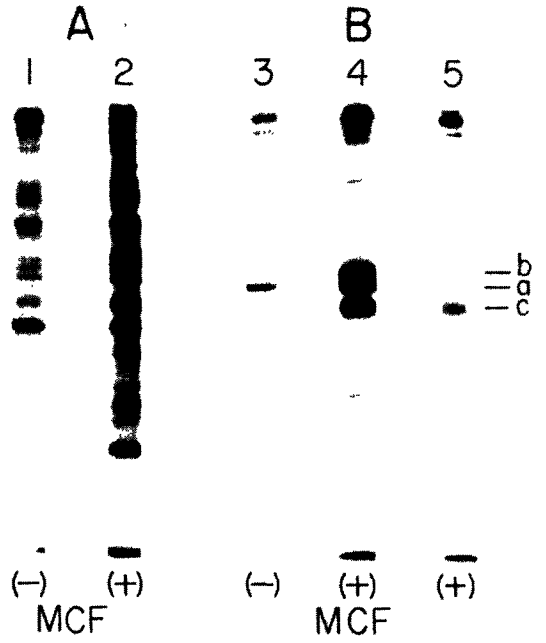

Fig.2. Effect of MCF on incorporation of [^{35}S]methionine into procollagenase and other proteins analyzed by SDS-PAGE and fluorography. Cells were incubated for 48 h with (+) (lanes 2,4,5) or without (−) (lanes 1,3) MCF, following which [^{35}S]methionine was added as described. (A) Total medium proteins. (B) Proteins complexed to antihuman collagenase immunoglobulin (lanes 3,4) or nonspecific immunoglobulin (lane 5). In medium from the MCF-stimulated cultures the major complexed proteins had M_r ~55000 (a) in addition to other components with M_r ~61000 (b) and ~49000 (c). A marked increase in labeling of these proteins was observed in medium from cultures preincubated with MCF.

procollagenase are shown in fig.2B. There was a marked enhancement of the labeling of proteins complexed with the antibody from the medium of cells incubated with MCF.

The major labeled band was of M_r ~55000 in addition to a more slowly migrating band of M_r ~61000. Another minor species was also seen of M_r ~45000; the intensity of labeling of this band was variable, but it appeared to be more prominent in samples stored for long periods even at −20°C. The direct addition of Trasylol (aprotinin), a serum protease inhibitor, to synovial cell cultures did not change the relative labeling of the proteins in the range M_r ~55000–61000, suggesting that proteolytic cleavage by serine proteases was not responsible for the multiplicity of proteins of this apparent size (not shown).

To investigate the possibility that the several proteins complexed by the collagenase antibody could result from the presence of glycosylated forms of the enzyme, medium from synovial cell cultures stimulated with MCF was chromatographed on Con A-Sepharose. The results are shown in fig.3. Several proteins did not bind to the Con A-Sepharose column whereas others were bound and could be eluted with 0.5 M α-methyl mannoside. When the bound and unbound fractions were incubated with the anticollagenase antibody and analyzed by SDS-PAGE it was found that the major procollagenase species of M_r ~55000 did not bind to the Con A-Sepharose column, whereas the minor species of M_r ~61000 did (fig.3B). These results are consistent with the interpretation that the major secreted procollagenase from stimulated and unstimulated human synovial cells is not glycosylated, but that the minor higher-M_r species is glycosylated. The apparent heterogeneity in the labeling of the procollagenase is therefore due, at least in part, to varying degrees of glycosylation.

Further evidence for glycosylation of the procollagenase was obtained by studies utilizing tunicamycin. As shown in fig.4A, in synovial cell cultures incubated with or without MCF, the simultaneous presence of tunicamycin resulted in a general shift of bands to those with lower M_r as well as a mild decrease in synthesis of all proteins. In cultures treated with tunicamycin, however, a decrease in the M_r ~61 kDa procollagenase species complexed with antibody was accompanied by an

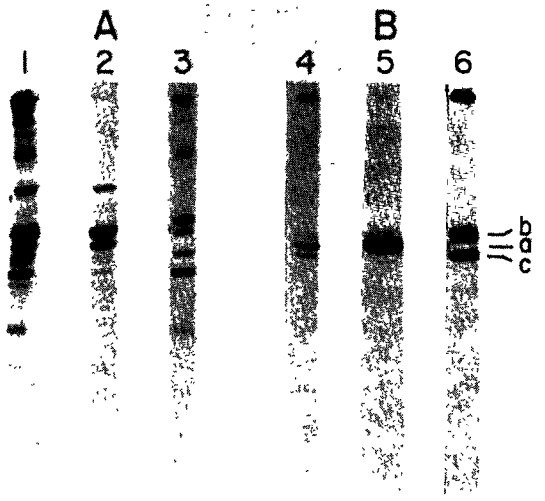

Fig.3. Binding to Con A-Sepharose of labeled proteins from the medium of synovial fibroblasts incubated with MCF, analysed by SDS-PAGE. Conditions were as described in section 2. Pattern of labeled proteins before addition to Con A-Sepharose (lane 1,4), fractions which did not bind to Con A-Sepharose (lanes 2,5) and fractions which bound and could be eluted with 0.5 M α-methylmannoside (lanes 3,6). (A) Samples analyzed by SDS-PAGE without incubation with antibody. (B) Samples complexed with antihuman collagenase immunoglobulin. Note that the major protein complexed by the antihuman collagenase immunoglobulin, of M_r ~55000 (a), did not bind to Con A-Sepharose, whereas the components of M_r ~61000 (b) and ~49000 (c) were bound and eluted with 0.5 M α-methylmannoside.

increase in the amount of ~55 kDa procollagenase species (fig.4B). These observations suggest that the ~55 kDa form is a precursor of a ~61 kDa glycosylated form. The apparently glycosylated form of the procollagenase comprised ~23–25% of the total labeled collagenase as estimated by cutting appropriate sections of the SDS-PAGE gel, extracting and counting the radioactivity. When the activity of nonglycosylated and glycosylated forms of the enzyme in medium from cells not incubated with [^{35}S]methionine was measured in a collagen fibril assay [4], the glycosylated form accounted for ~25–28% of the total collagenase activity (not shown).

Experiments were next designed to determine the kinetics of the MCF effect on stimulation of procollagenase synthesis. In medium from cells prein-

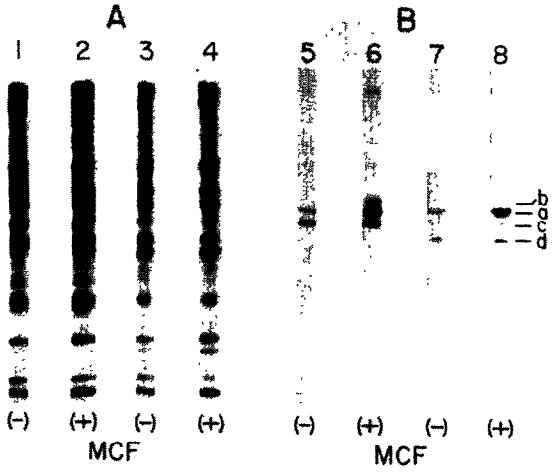

Fig.4. Effects of tunicamycin on proteins synthesized by rheumatoid synovial fibroblasts incubated with or without MCF. Cells were incubated for 48 h with (+) (lanes 2,4,6,8) or without (−) (lanes 1,3,5,7) MCF. Tunicamycin was then added to samples (shown in lanes 3,4,7,8) at 44 h. [^{35}S]Methionine was then added at 48 h to all samples and incubation continued for 24 h. Medium proteins were then analyzed by SDS-PAGE. (A) Patterns of labeled medium proteins from cell-free medium of samples not incubated with antibody. (B) Corresponding samples as in A, but complexed with antihuman collagenase immunoglobulin. Note that the major protein complexed by the antihuman collagenase immunoglobulin of M_r ~55000 (a) persisted after incubation with tunicamycin whereas the protein of M_r ~61000 (b) could no longer be detected. The protein of M_r ~49000 (c) decreased and a new band appeared at M_r ~41000 (d).

cubated with MCF, procollagenase in the medium was detected within 2 h after the addition of radioactive methionine, as shown in fig.5. The rate of synthesis of procollagenase appeared to fall off by 24 h of incubation. These observations are consistent with little or no intracellular storage of the newly synthesized procollagenase in these human synovial cells. The increase in labeling of the major 55 kDa procollagenase protein by cells incubated with MCF ranged from 12- to 14-fold over that of cells incubated with DME medium alone at 2, 4 and 8 h after addition of [^{35}S]methionine (fig.5). When MCF and [^{35}S]methionine were added simultaneously to synovial fibroblast cultures, significant amounts of labeled complexed protein were not detected until 18 h later (not shown).

4. DISCUSSION

We have previously demonstrated that MCF/IL1 increases the levels of collagenase activity (measured following activation of latent enzyme with trypsin) in medium from human rheumatoid synovial fibroblast cultures [1,5,6–9]. The present results indicate that MCF/IL1 increases the synthesis of procollagenase. Incubation with the MCF preparation used here also increased [^{35}S]-methionine incorporation into several other proteins with M_r ranging from ~40000 to 70000 the identity of which has yet to be established. We have also observed in other studies that MCF increases the synthesis of several macromolecules such as type I and III procollagens and fibronectin [12].

The exact mechanism of the MCF/IL1 effect to increase procollagenase levels remains to be elucidated. In other systems in which collagenase synthesis has been studied such as cultures of rabbit synovial cells, the addition of phorbol myristate acetate or monosodium urate crystals increases procollagenase protein synthesis as well as the level of translatable hybridizable procollagenase mRNA consistent with transcriptional control of pro-

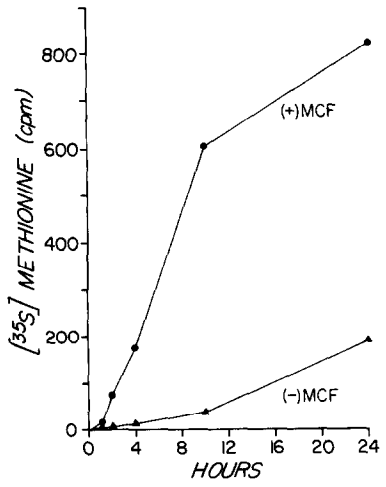

Fig.5. Time course of effect of MCF preincubation on labeling of major procollagenase protein. Conditions were those described in fig.2, except that samples were removed at indicated times and proteins resolved by SDS-PAGE. The band corresponding to the major procollagenase species (M_r ~55000) was cut from the dried gel, extracted with NCS solubilizer and counted in a liquid scintillation counter.

collagenase synthesis [18–21]. The present results would be compatible with such a control mechanism. Although only partially purified preparations of MCF/IL1 were used here, it has now been shown that at least one recombinant DNA form of IL1 [22] increases medium levels of collagenase activity [23]. It is therefore likely that the stimulatory effects observed here are due to IL1.

The antibody to human skin fibroblast procollagenase used here complexed predominantly 2 forms of human rheumatoid fibroblast procollagenase, with M_r ~55000 and ~61000, both of which were active enzymatically. These 2 forms could be separated on Con A-Sepharose columns. The major ~55 kDa species, comprising approx. 75% of the total procollagenase, appeared to be nonglycosylated. The minor species, whose labeling was abolished by the addition of tunicamycin, was likely a glycosylated form of the ~55 kDa precursor. Similar conclusions were derived from observations of cultured rabbit synovial fibroblasts [21]. MCF/IL1 increases the synthesis of both forms of the enzyme. This increase in synthesis in cells preincubated with IL1 was detected within 2 h of addition of the labeled amino acid. The monokine MCF/IL1 thus has several profound effects on the function of the synovial fibroblasts. It remains to be shown how this stimulation of procollagenase synthesis is mediated.

ACKNOWLEDGEMENTS

We are grateful to Drs Mary L. Stephenson and Edward D. Harris jr for helpful discussions, to Merrilee Roelke and Marlena Suddard for help in cell cultures and to Michele Angelo for preparation of the manuscript. This work was supported by USPHS grants AM-035564 and AM-07258. P.A.McC. was recipient of a Postdoctoral Fellowship of the Arthritis Foundation. This is publication 987 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities.

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