Translational augmentation of pro-matrix metalloproteinase 3 (prostromelysin 1) and tissue inhibitor of metalloproteinases (TIMP)-1 mRNAs induced by epidermal growth factor in human uterine cervical fibroblasts

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Abstract The mechanisms by which epidermal growth factor (EGF) enhances the production of pro-matrix metalloproteinase 3 (proMMP-3/prostromelysin 1) and tissue inhibitor of metallo-proteinases (TIMP)-1 were investigated using human uterine cervical fibroblasts. The treatment of the cells with EGF for 24 h resulted in about 5–6-fold increase in the production of proMMP-3 and TIMP-1 compared with the untreated control cells. This increase was accompanied by an increase of proMMP-3 and TIMP-1 mRNAs. However, an about 3- and 2-fold increase in the production of proMMP-3 and TIMP-1, respectively, was observed as early as 1 h after the treatment of the cells with EGF, and it was not accompanied by any apparent increase in proMMP-3 and TIMP-1 mRNAs. This early effect of EGF on the enhanced production of proMMP-3 and TIMP-1 was not inhibited by actinomycin D, even though actinomycin D inhibited the synthesis of the total RNA in both the EGF-treated and untreated cells. These results indicate that EGF enhances the apparent production of proMMP-3 and TIMP-1 by two mechanisms: one by the accelerated translation of their mRNAs; and the other by the enhanced transcription of their genes. The former event takes place much earlier than the latter.

Key words: Matrix metalloproteinase 3; Prostromelysin 1; Tissue inhibitor of metalloproteinases-1; Epidermal growth factor; Human uterine cervical fibroblast

1. Introduction

Matrix metalloproteinases (MMPs) are considered to play an important role in the degradation of connective tissue matrix components in both physiological and pathological processes such as in wound healing, embryogenesis, angiogenesis, rheumatoid arthritis, tumor cell invasion and metastasis [1,2]. It is well-recognized that a number of cytokines and growth factors, e.g. IL-1, TNFα, transforming growth factor β and EGF, participate in the regulation of production of MMPs

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and their inhibitors (TIMPs) in connective tissue cells [3–6], and the overall imbalance between MMPs and TIMPs is likely to be a key determinant of connective tissue matrix catabolism under physiological and pathological conditions [7]. All these cytokines and growth factors regulate the transcription of a number of MMP genes (see [1] for a review). For example, EGF augments the production of proMMP-1 (interstitial pro-collagenase), proMMP-3 (prostromelysin 1) and TIMP-1 [8] along with an increase of their mRNAs [8–10]. McDonnell et al. [11] have reported that the EGF-induced gene expression of proMMP-3 requires activation of protein kinase C and induction of proto-oncogenes, c-*fos* and c-*jun*. The induced c-Fos and c-Jun proteins form a Fos-Jun heterodimer which initiates the transcription of the proMMP-3 gene through the its binding to TRE/AP-1-binding site [12–14]. Similarly, the induction of proMMP-1 and proMMP-3 transcripts by phorbol myristate acetate [15], IL-1 [16], and TNFα [17] is thought to be in part mediated by AP-1. However, an increase of proMMP-3 transcripts requires several hours after treating with EGF or with other stimuli [11].

In this report, we have investigated the production of proMMP-3 and TIMP-1 at much earlier time points (within 1 h) after EGF treatment using human uterine cervical fibroblasts, and observed that the synthesis of these gene products was enhanced well before an increase of their mRNAs was detected. Our studies suggest that the initial augmentation in proMMP-3 and TIMP-1 production by EGF results from accelerated translation of their mRNAs.

2. Materials and methods

2.1. Materials

The following reagents were obtained commercially: MEM was from Grand Island Biochemical, Grand Island, NY. FBS was from Whittaker, Walkersville, MD. Actinomycin D-mannitol was from Sigma, St Louis, MO. Horseradish peroxidase-conjugated donkey anti-(sheep IgG-(H+L))IgG was from The Binding Site, Birmingham, UK. The ECL-Western blotting detection reagents were from Amersham, Tokyo, Japan. hEGF was from PROGEN Biotechnik, Heidelberg, Germany. [5-³H]uridine was from American Radiolabeled Chemicals, St Louis, MO. Sheep anti-(human MMP-3) antibody and sheep anti-(human TIMP-1) antibody were prepared as described previously [18,19]. A 1.4-kb cDNA clone encoding human MMP-3 was isolated from the λgt11 cDNA library of human rheumatoid synoviocytes. The cDNA sequence was confirmed by the dideoxy chain termination method. TIMP-1 cDNA (0.7 kb) was kindly provided by Dr. M. Naruto at Basic Research Laboratories, Toray Industries, Kamakura, Kanagawa, Japan. GAPDH cDNA (1.1 kb) was from CLONTECH Laboratories, Palo Alto, CA. Other reagents used were the same as in the previous paper [20].

2.2. Cell culture

Preparation and maintenance of human uterine cervical fibroblasts were the same as described previously [20]. For most experiments, the cells up to the 10th passage in a 12-well plate were used. After confluence, the cells were cultured without serum for 24 h and then treated with hEGF in the serum-free MEM/0.2% (w/v) LAH to stimulate the production of proMMP-3 and TIMP-1. All experiments were conducted at least in duplicate.

2.3. ECL-Western blotting for proMMP-3 and TIMP-1

ProMMP-3 and TIMP-1 in the culture media were analyzed by Western blotting. Each sample from triplicate wells was concentrated by precipitating the proteins with final 3.3% (w/v) trichloroacetic acid and then subjected to SDS–PAGE with 11.5% (w/v) acrylamide gel under reducing conditions [21]. After electrophoresis, proteins in the gel were electrotransferred onto a nitrocellulose filter. The filter was reacted with sheep anti-(human MMP-3) or sheep anti-(human TIMP-1) antibody, which was then complexed with the horseradish peroxidase-conjugated donkey anti-(sheep IgG(H + L))IgG. Immunoreactive proMMP-3 and TIMP-1 were indirectly visualized using the ECL-Western blotting detection reagents according to the manufacturer's instructions. The amounts of immunoreactive antigens were determined by densitometric analysis.

2.4. Extraction of RNA, slot–blot analysis and Northern blotting

Total cytoplasmic RNA of cultured human uterine cervical cells was isolated using a commercial RNA extraction kit (Isogen; Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Total RNA was denatured with formaldehyde and then transferred onto a nylon membrane. Slot-blot analysis was carried out by the method of Wahl [22]. Membrane was hybridized with ^{32}P-labeled nick-translated proMMP-3, TIMP-1 and β-actin cDNAs at 42°C in 10×Denhardt's solution, 5×SSPE (1×SSPE is 0.18 M NaCl/0.01 M NaH$_2$PO$_4$/1.1 mM disodium EDTA) containing 0.1% (w/v) SDS, and 150 µg of heat-denatured salmon sperm DNA/ml. After hybridization, the membrane was washed twice in 1×SSC (0.15 M NaCl/0.015 M sodium citrate, pH 7.0)/0.1% (w/v) SDS, and then washed in 0.1×SSC/0.1% (w/v) SDS at room temperature for 30 min. The radioactivity was measured using Bio-imaging analyzer BAS 2000 (Fuji Photo Film, Tokyo, Japan).

Northern blotting was also carried out as follows. Each total RNA which was denatured with formaldehyde and formamide, was run on formaldehyde-agarose gels and then transferred onto a nylon membrane. Membrane was hybridized with ^{32}P-labeled random primed proMMP-3, TIMP-1 and GAPDH cDNAs at 42°C in a solution containing 5×SSC, 1×Denhardt's solution, 50 mM sodium phosphate (pH 6.5), 0.1% (w/v) SDS, 50% formamide and 200 µg of heat-denatured salmon sperm DNA/ml. After hybridization, the membrane was washed for 10 min at room temperature in 2×SSC/0.1% (w/v) SDS and exposed to Konica X-ray film/medical at −80°C.

2.5. Measurement of the synthesis of total RNA

Confluent human uterine cervical cells in 12-well plates were deprived from serum for 24 h, and then incubated with [5-^{3}H]uridine (37 kBq/ml) in MEM/0.2% (w/v) LAH under presence or absence of hEGF (100 ng/ml). After pulse-labeling for 1 h, the culture media were removed from the 12-well plates. The cells were washed twice with calcium and magnesium-free PBS, and treated with ice-cold 10% (w/v) trichloroacetic acid for 30 min. The cells were solubilized with 0.5 N NaOH, and the radioactivity in the solution was then measured.

3. Results and discussion

EGF regulates various cellular events in many cell types. For example, augmentation of the production of proMMPs and TIMP-1 in human and rat fibroblasts is closely correlated to connective tissue matrix remodeling [8–11]. We investigated the mechanisms by which EGF enhanced the production of proMMP-3 and TIMP-1 using human uterine cervical fibroblasts.

When human uterine cervical fibroblasts were treated with hEGF for 24 h, the accumulation of proMMP-3 and TIMP-1

in the culture media increased in a dose-dependent manner (1–100 ng/ml) (data not shown) as reported for MRC-5 human fibroblasts [10]. We then examined the time course of proMMP-3 and TIMP-1 production after the treatment of the cells with hEGF at a concentration of 100 ng/ml. As shown in Fig. 1, there was an about 2–3-fold increase in the production of both components as early as 1 h after hEGF treatment when compared with the untreated control cells (panel A), and it continued up to 24 h (panel B). An enhancement of specific protein synthesis in response to cytokines or growth factors is usually paralleled with an increase in mRNA; this applies to the regulation of proMMP-3 and TIMP-1 in many cell types [1,23]. We, therefore, examined the levels of proMMP-3 and TIMP-1 mRNAs after treating the cells with hEGF (100 ng/ml), but an increase of these mRNAs was first detected at 6 h and reach to a steady-state level after 8 h (Fig. 2). In a separate experiment, we also observed that an increase in incorporation of [^{3}H]uridine into the total RNA was first detected at 6 h after hEGF treatment (data not shown). Fig. 3 compares the time-dependent increase of proMMP-3 and TIMP-1 protein production and their mRNA levels. It is noteworthy that there was a 2–3-fold increase in the production of both proMMP-3 and TIMP-1 in the hEGF-treated cells at 1-, 2- and 4-h time points when compared with the control cells, but there was no detectable increase in their mRNAs. The levels of proMMP-3 and TIMP-1 proteins produced by the hEGF-treated cells after 12 h and 24 h were 3–4- and 5–6-fold higher than those of the control cells, respectively, where their mRNA levels increased only 1.7–1.9-fold (Fig. 3). These observations suggest that the increase of Prommp-3 and TIMP-1 production during the first 4 h of EGF treatment was due to the enhanced translation of their preexisting mRNAs at a low level and the latter production (after 6 h) were reflected from
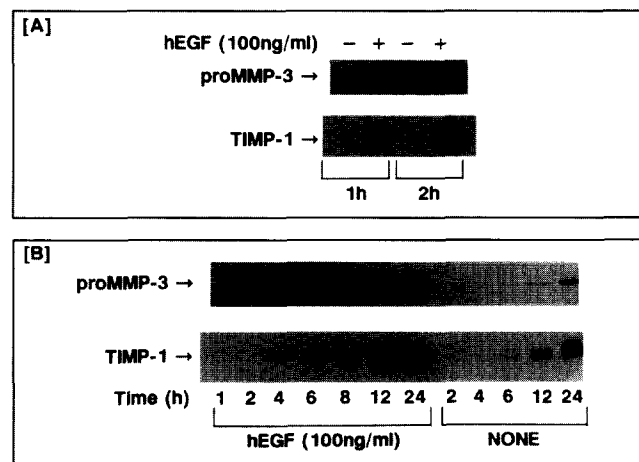

Fig. 1. Time-dependent effects of EGF on the production of proMMP-3 and TIMP-1 in human uterine cervical fibroblasts. Confluent uterine cervical fibroblasts at the 5th passage in 12-well plates were deprived from serum for 24 h prior to the stimulation with or without hEGF (100 ng/ml) in 0.2% (w/v) LAH/MEM. At the times indicated, culture media (3 ml for panel A; 1 ml for panel B) were harvested and the proteins precipitated by mixing with 1/5 vol. of 20% (w/v) trichloroacetic acid. The precipitates collected by centrifugation were dissolved in reducing SDS-PAGE sample buffer and subjected to ECL-Western blotting. Immunoreactive proMMP-3 and TIMP-1 were detected as described in section 2.

both the increased steady-state levels of their mRNAs as well as translational augmentation.

To further investigate the possible translational enhancement that was observed at an earlier time point after EGF treatment, we treated the cells first with actinomycin D for 1–4 h and then with hEGF (100 ng/ml) in the presence of actinomycin D for 1 h, and examined the changes in levels of proMMP-3 and TIMP-1 proteins in paralleled with their mRNAs. As shown in Fig. 4A, pretreatment of the cells with actinomycin D (1 μM) did not alter the hEGF-mediated production of proMMP-3 (2–3-fold) and TIMP-1 (4–5-fold). Furthermore, hEGF did not modulate the steady-state levels of proMMP-3 and TIMP-1 mRNAs either in the presence or the absence of actinomycin D at the concentration of 1 μM (Fig. 4B). The mRNA levels of both proMMP-3 and TIMP-1 in the control and the actinomycin D-treated cell were almost constant during the total 5-h treatment. On the other hand, RNA synthesis of the control and the hEGF-treated cells was completely inhibited by actinomycin D (Fig. 5). No significant decrease of the steady-state levels of proMMP-3 and TIMP-1 mRNAs during a 5-h actinomycin D treatment is supported by relatively long half-lives of their mRNAs (16–60 h for proMMP-3 and >60 h for TIMP-1) [24,25]. These results further support the notion that the enhanced production of proMMP-3 and TIMP-1 during the first 1-h treatment with EGF is independent of transcriptional activity. The increased production of proMMP-3 and TIMP-1 is also unlikely to be due to the stimulation of the secretion of these proteins since both proteins do not accumulate intracellularly [20,26].

Delany and Brinckerhoff [24] reported that the EGF-mediated enhanced production of proMMPs-1 and -3 in human foreskin fibroblasts is in part regulated posttranscriptionally; i.e. EGF stabilizes the proMMP mRNAs, which in turn augments the synthesis of these proteins when measured after a 12-h treatment. Our present studies, however, demonstrated that the steady-state levels of proMMP-3 and TIMP-1 mRNAs did not change at least during the first 1-h treatment with hEGF with and without actinomycin D treatment, indicating that the mRNA stabilization effect was not observed

Fig. 3. Time-dependent increase of proMMP-3 and TIMP-1 proteins and their mRNAs. Time-dependent increase in the relative amounts of proMMP-3 protein (panel A) and TIMP-1 protein (panel C) and that of the corresponding mRNA levels (panels B and D) obtained from Figs. 1 and 2 were plotted. ProMMP-3 and TIMP-1 were not detected in the control cells at a 1-h time point by the method used. Thus, the relative protein levels were calculated as an fold-increase taking the 2-h time point of the control cells as 1. The relative mRNA levels were standardized by β-actin taking the value of the control cells as 1.

during a short period of EGF treatment. Thus, we conclude that the increase in proMMP-3 and TIMP-1 production at the earlier phase of EGF treatment most likely results from the enhanced translation of their messages. Similar enhanced translational effects of EGF were also observed with human dermal fibroblasts (data not shown).

We also found that IL-1α similarly augmented the production of proMMP-3 and TIMP-1 without changing their mRNA levels in human uterine cervical cells (T. Hosono, A. Ito and Y. Mori, unpubl. results). When the inductive effects of hEGF and IL-1α were compared at the same molar concentration, the rate of augmentation of proMMP-3 and TIMP-1 by hEGF was twice as fast as that by IL-1α. Therefore, the translational activation of proMMP-3 and TIMP-1 mRNAs appears to be a common phenomenon for these two factors, but EGF is more effective than IL-1α.

It is not clear how EGF accelerates the translation of proMMP-3 and TIMP-1 mRNAs in human uterine cervical and dermal fibroblasts. The translational rate of some mRNAs is controlled by the phosphorylation of various cellular proteins, especially eukaryotic initiation factor-4F (eIF-4F) and ribosomal S6 protein [27]. These eIF-4F and ribosomal S6 proteins are phosphorylated by protein kinase C (PKC) and S6 kinase in rabbit reticurocytes and mouse 3T3-L1 cells, respectively, and they closely participate in the translation of mRNAs for protein synthesis [28,29]. In this point of view, it is of interest that EGF activates PKC [11,30] and S6 kinase [31]. Thus, it is tempting to speculate that EGF may accelerate the translation of proMMP-3 and TIMP-1 by in part activating these kinases. Indeed, we observed that a protein kinase inhibitor, 1-(5-isoquinolinesulfo-nyl)-2-methylpiperazine dihydrochloride (H7) diminished the enhanced production of proMMP-3 and TIMP-1 induced by a 2-h treatment with hEGF (T. Hosono, A. Ito and Y. Mori, unpubl. results).

In conclusion, we have demonstrated that the apparent augmentation of production of proMMP-3 and TIMP-1 in

ProMMP-3 **TIMP-1** **β-actin**

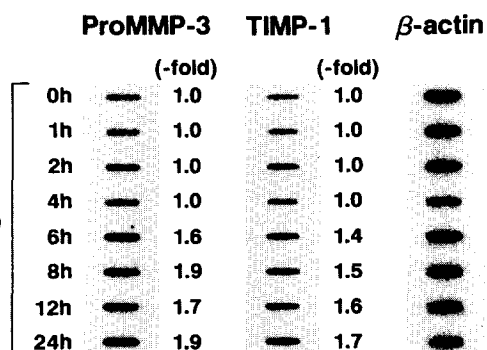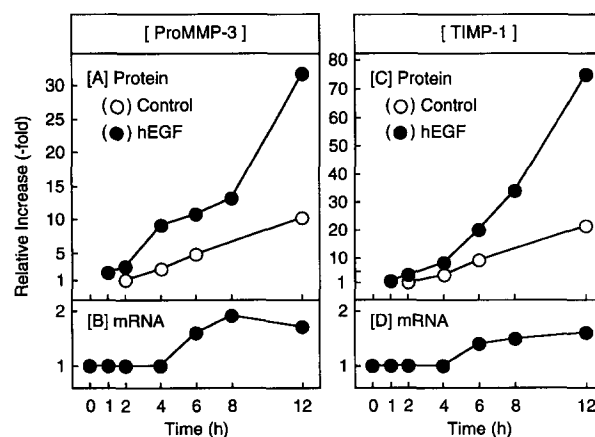|		ProMMP-3 (-fold)	TIMP-1 (-fold)	β-actin
	0h	1.0	1.0	
	1h	1.0	1.0	
	2h	1.0	1.0	
hEGF (100ng/ml)	4h	1.0	1.0	
	6h	1.6	1.4	
	8h	1.9	1.5	
	12h	1.7	1.6	
	24h	1.9	1.7	

Fig. 2. Effects of EGF on the steady-state levels of proMMP-3 and TIMP-1 mRNAs in human uterine cervical fibroblasts. Confluent uterine cervical fibroblasts at the 10th passage in 100-mm diameter dishes were treated as in Fig. 1. At the time indicated, the total RNA was extracted and 5 μg of the total RNA was applied to a nylon membrane using a slot–blot apparatus as described in section 2. The relative mRNA levels were determined by scanning of the radioactivity of each band with Bio-imaging analyzer BAS 2000 and standardizing them by β-actin. The value of the control cells was taken as 1.
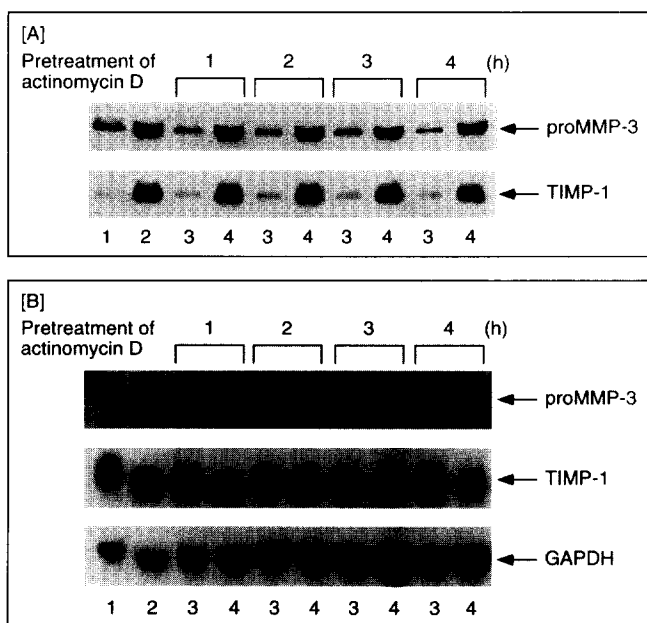
Fig. 4. Effects of actinomycin D on the production of proMMP-3 and TIMP-1 and on the steady-state levels of their mRNAs in human uterine cervical fibroblasts during the early EGF treatment. Confluent uterine cervical fibroblasts at the 8th passage in 100-mm diameter dishes were first treated with actinomycin D (1 μM) for the indicated periods and then cotreated with hEGF and actinomycin D for 1 h. The amounts of proMMP-3 and TIMP-1 in the culture media were analyzed by ECL-Western blotting, and their mRNAs in 30 μg of the total RNA were monitored by Northern blotting as described in section 2. Panel A; protein levels of proMMP-3 and TIMP-1 and panel B; their mRNA levels. Lane 1, control cells; lane 2, cells treated with hEGF (100 ng/ml); lane 3, control cells pretreated with actinomycin D (1 μM) at indicated periods and then additional 1 h; and lane 4, cells pretreated with actinomycin D as lane 3 and then cotreated with hEGF (100 ng/ml) and actinomycin D (1 μM) for 1 h.
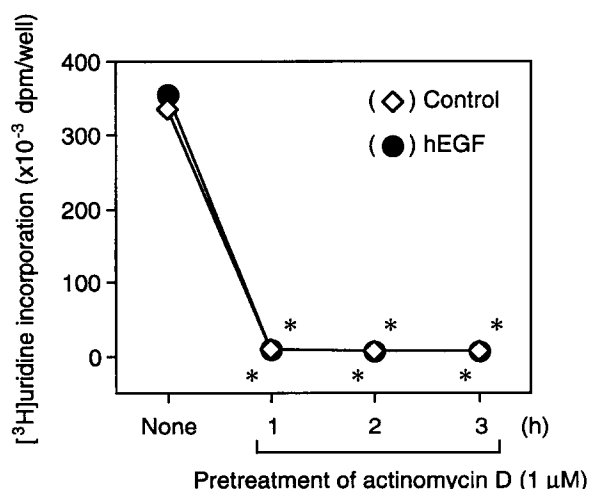

Fig. 5. Effect of actinomycin D on the synthesis of total RNA in human uterine cervical fibroblasts during the early EGF treatment. Confluent uterine cervical fibroblasts at the 8th passage in 12-well plates were first treated with actinomycin D (1 μM) for the indicated periods (1–3 h), and then cotreated with hEGF (100 ng/ml) and actinomycin D (1 μM) in 0.2% (w/v) LAH/MEM containing [5-^{3}H]uridine (37 kBq/ml) for 1 h. The synthesis of total RNA was measured as described in section 2. Each point represents the mean ± S.D. from three individual wells. *Significantly different from the cells without treatment of actinomycin D ($P<0.001$).

human uterine cervical cells by EGF is finely controlled via two different mechanisms. One is a well-recognized transcriptional mechanism by which EGF increases the steady-state levels of their mRNAs. The other mechanism lies on the enhancement of the translational rate of proMMP-3 and TIMP-1 mRNAs. The latter mechanism may in part explain relatively higher production rate of these proteins compared with a limited increases in their mRNAs.

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