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FEBS Letters 341 (1994) 109–112

FEBS 13772

FEBS LETTERS

Progesterone increases the production of tissue inhibitor of metalloproteinases-2 in rabbit uterine cervical fibroblasts

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Received 1 February 1994

Abstract

Rabbit uterine cervical fibroblasts in culture produces tissue inhibitor of metalloproteinases (TIMP)-1 and TIMP-2. When cells were treated with physiological concentrations of progesterone, the production of two TIMPs increased, and essentially all TIMP-2 was found to be complexed with promatrix metalloproteinase 2 (proMMP-2)/progelatinase A. Progesterone did not modulate the production of proMMP-2 and resulted in the increased total amount of proMMP-2-TIMP-2 complex. These observations provide the first evidence that progesterone participates in maintaining the homeostasis of connective tissue matrix in uterine cervix by augmenting both TIMP-1 and TIMP-2 production along with the known suppressive effects on the proMMP-1 and proMMP-3 production.

Key words: Progesterone; TIMP-2; Gelatinase A; Matrix metalloproteinase; Uterine cervix

1. Introduction

Matrix metalloproteinases (MMPs) are a group of related enzymes that participates in pathological and physiological breakdown of extracellular matrix components such as in arthritis, cancer cell invasion and metastasis, and cervical ripening and dilation at term pregnancy [1–6]. The activities of MMPs in the tissue are regulated by a number of factors, including gene expression of MMPs, extracellular activation of MMPs from their precursors (proMMPs) and inhibition of MMPs by endogenous tissue inhibitors of metalloproteinases (TIMPs) [1,2]. In addition, the expression of TIMPs is shown to be regulated by a number of cytokines, hormones, transforming growth factor, and phorbol esters [7–9]. Recent findings of specific complex formation between proMMP-2 (progelatinase A) and TIMP-2 [10–13], and between proMMP-9 (progelatinase B) and TIMP-1 [14,15] have introduced additional complexity in regulation of extracellular matrix catabolism by MMPs.

We have previously reported that in rabbit uterine cervical fibroblasts the production of TIMP-1 is up-regulated by progesterone or oestradiol-17β whereas the production of proMMP-1 (interstitial procollagenase) and proMMP-3 (prostromelysin 1) is suppressed. These observations suggest that matrix catabolism in uterine cervix is down-regulated by these hormones [9]. The eventual catabolism of matrix, however, is dependent on the balance between the overall production of MMPs and TIMPs. In this regard, little is known about the regulation of TIMP-2 production, although it has been reported that TIMP-1 and TIMP-2 are differently regulated [16,17]. Here, we report that progesterone enhances the production of TIMP-2 in rabbit uterine cervical fibroblasts as well as TIMP-1, resulted in accumulation of the proMMP-2-TIMP-2 complex in the medium of the progesterone-treated cells. The results suggest that progesterone is a negative regulator of extracellular matrix degradation in uterine cervix.

2. Materials and methods

2.1. Materials

The following reagents were obtained commercially: Eagle's minimum essential medium (MEM) from Grand Island Biological Co., Grand Island, NY, USA; fetal-bovine serum (FBS) from Whittaker Bioproducts, Walkersville, MD, USA; progesterone, oestradiol-17β, alkaline phosphatase-conjugated donkey anti-(sheep IgG) IgG and alkaline-phosphatase conjugated goat anti-(rabbit IgG) IgG from Sigma Chemical Co., St. Louis, MO, USA; Gelatin-Sepharose 4B from Pharmacia LKB Biotechnology, Uppsala, Sweden. Human TIMP-2 was purified from the culture medium of human uterine cervical fibroblasts by chromatography on Gelatin-Sepharose as proMMP-2-TIMP-2 complex and subsequent separation of the complex by SDS-PAGE under non-reducing condition. After SDS-PAGE, TIMP-2 was electroeluted from the gel. Human TIMP-1 was also purified by the method of Morodomi et al. [18]. Sheep anti-(human TIMP-1) antiserum, sheep

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Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; MEM, Eagle's minimum essential medium; LAH, lactalbumin hydrolysate; FBS, fetal bovine serum; PBS(−), calcium and magnesium-free phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

SSDI 0014-5793(94)00157-Q

anti-(human TIMP-2) antiserum and rabbit anti-(human proMMP-2) antiserum were prepared by injecting the purified antigens emulsified with complete Freund's adjuvant and the subsequent boosters with incomplete Freund's adjuvant as described previously [19]. Other reagents used were of analytical-reagent grade.

2.2. Cell culture of rabbit uterine cervix

Uterine cervical fibroblasts were established from Nippon white rabbits of 23 days gestational age and maintained in culture in 1.0 ml of MEM/10% (v/v) FBS in 24-multiwell plates as described previously [20]. In all experiments, cells at the first passage were used. To estimate production of proMMP-2, TIMP-1 and TIMP-2, the culture medium was changed to MEM/0.2% (w/v) lactalbumin hydrolysate (LAH) after confluence, and progesterone and other reagents were added to the medium as an ethanol solution. The final ethanol concentration was 0.1% (v/v) in all cultures, and the same amount of vehicle was added to the control cultures. The harvested culture media were stored at −20°C until use.

2.3. Reverse gelatin zymography for TIMPs

The sample was subjected to SDS-PAGE [21] using a 12.5% (w/v) acrylamide slab gel containing 0.6 mg/ml gelatin under non-reducing conditions. Then SDS in the gel was removed by rinsing with 50 mM Tris-HCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.02% NaN_3 (pH 7.5) containing 2.5% (w/v) Triton X-100. The gel was then incubated in 20 ml of the same buffer without Triton X-100 and containing 5 units/ml of MMP-2 for 20 h at 37°C. The gel was stained with 0.1% (w/v) Coomassie brilliant blue in 50% (v/v) methanol/20% (v/v) acetic acid, and destained with 1% (v/v) formic acid/30% (v/v) methanol.

2.4. Western blotting for proMMP-2, TIMP-1 and TIMP-2

Each sample from quadruplicate wells was mixed with 1/5 volume of 20% (w/v) trichloroacetic acid. The resultant precipitates were dissolved in reducing SDS-PAGE sample buffer [21], and the portion was first subjected to SDS-PAGE using 10% (w/v) (for proMMP-2) and 12.5% (w/v) (for TIMP-1 and TIMP-2) acrylamide slab gel under reducing conditions, and then proteins in gels were electro-transferred onto a nitrocellulose membrane. The membrane was reacted with sheep anti-(human TIMP-1 or TIMP-2) antiserum which was then complexed with alkaline phosphatase-conjugated donkey anti-(sheep IgG) IgG. Immunoreactive TIMP-1 and TIMP-2 were visualized indirectly using 5-bromo-4-chloro-3-indolyl phosphate and Nitroblue tetrazolium as described previously [8]. ProMMP-2 was similarly detected using rabbit anti-(human proMMP-2) antiserum and alkaline phosphatase conjugated goat anti-(rabbit IgG) IgG.

2.5. Identification of the proMMP-2 complex with TIMP-2

An aliquot of the culture media (1.0 ml) was mixed with 100 μl of 50% (v/v) gelatin–Sepharose, and the mixture was incubated at room temperature for 45 min, and centrifuged at 11,750 × *g* for 10 min. The precipitates (gelatin-binding fraction) were washed twice with PBS(−), and then subjected to Western blotting for proMMP-2 and TIMP-2 as described above. The supernatant (gelatin-nonbinding fraction) was again treated with gelatin–Sepharose and centrifuged as above, and then the supernatant was also subjected to Western blotting.

3. Results and discussion

3.1. Effect of progesterone on the production of TIMP-2 in rabbit uterine cervical fibroblasts

Reverse gelatin zymographic analysis of culture medium of rabbit uterine cervical fibroblasts exhibited two inhibitory bands at around 28 kDa and 20 kDa, and the molecular masses corresponding to those of human TIMP-1 and human TIMP-2, respectively (Fig. 1, lane 1). The results indicate that rabbit uterine cervical fibroblasts spontaneously produce and secrete TIMP-2 as well as TIMP-1. When the confluent cells were treated

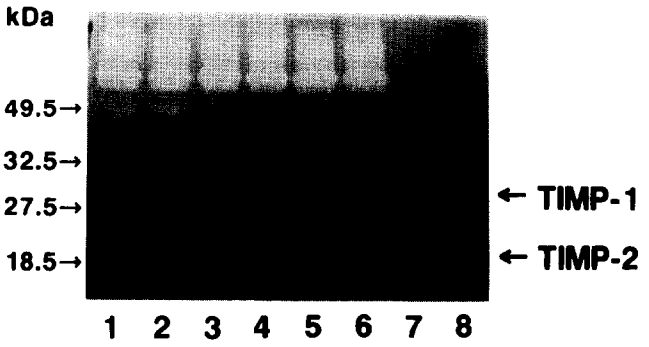

Fig. 1. Reverse gelatin zymogram for TIMPs from rabbit uterine cervical fibroblasts. Confluent rabbit uterine cervical fibroblasts were treated with various concentrations of progesterone in 1.0 ml of 0.2% (w/v) LAH/MEM for 48 h. An aliquot (10 μl) of the culture media from quadruplicate wells was subjected to reverse gelatin zymography as described in the text. Three independent experiments were highly reproducible and the typical data are shown. Lane 1, control; lanes 2–6, cells treated with progesterone at 1×10^{-10} M, 1×10^{-9} M, 1×10^{-8} M, 1×10^{-7} M and 1×10^{-6} M, respectively; lane 7, purified human TIMP-1 (1 ng) and lane 8, purified human TIMP-2 (4 ng).

with progesterone (1×10^{-10} M to 1×10^{-6} M) for 48 h, the accumulation of TIMP-1 and TIMP-2 in the culture media increased in a dose-dependent manner (Fig. 1, lanes 2–6). This was also supported by Western blotting: maximum 2-fold increase was observed with 1×10^{-6} M of progesterone as compared with the untreated control (Fig. 2A, lane 6). Progesterone augmented the production of TIMP-1 more effectively than that of TIMP-2: a maximum 9-fold increase of the basal level was detected with 1×10^{-6} M progesterone (Fig. 2B, lane 6). On the

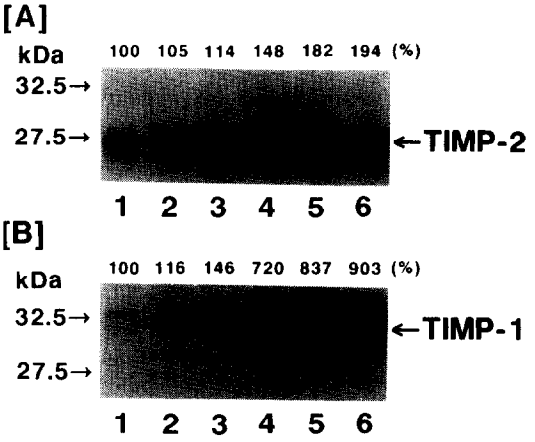

Fig. 2. Western blotting for TIMPs-1 and -2 in culture media of rabbit uterine cervical fibroblasts treated with progesterone. The harvested culture medium (1.2 ml) as described in Fig. 1 was concentrated and subjected to Western blotting for TIMP-1 and TIMP-2 as described in the text. The relative amounts of TIMP-1 and TIMP-2 were quantified by densitometric scanning, taking the control as 100%. Three independent experiments were highly reproducible and the typical data are shown. Lane 1, control and lanes 2–6, cells treated with progesterone at 1×10^{-10} M, 1×10^{-9} M, 1×10^{-8} M, 1×10^{-7} M and 1×10^{-6} M, respectively. Panel (A), TIMP-2 and panel (B), TIMP-1.

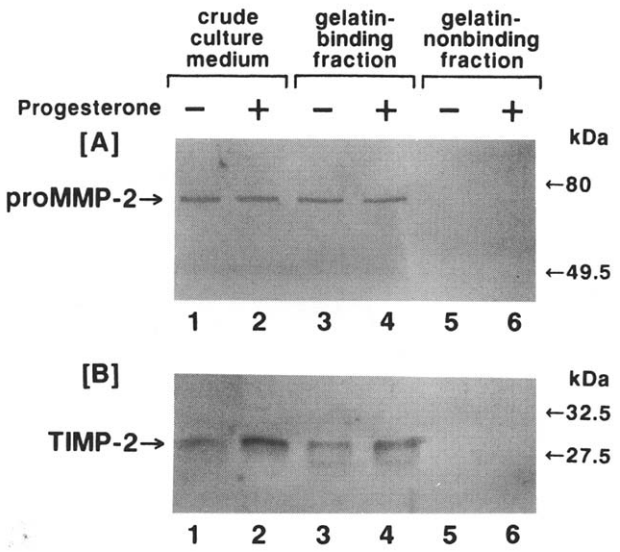

Fig. 3. Identification of the formation of proMMP-2 complex with TIMP-2 in rabbit uterine cervical fibroblasts. The harvested culture medium (1.0 ml) of rabbit uterine cervical fibroblasts treated with or without progesterone at 1×10^{-6} M as described in Fig. 1. was mixed with 100 μl of 50% (v/v) gelatin–Sepharose. ProMMP-2 and TIMP-2 in gelatin-binding fraction and gelatin-nonbinding fraction were analyzed by Western blotting as described in the text. Two independent experiments were highly reproducible and the typical data are shown. Panel (A), proMMP-2 and panel (B), TIMP-2.

contrary, oestradiol-17β failed to increase the production of TIMP-2 although it increased the production of TIMP-1, but to a lesser extent compared with progesterone (data not shown).

3.2. *Progesterone increased the complex of proMMP-2 and TIMP-2*

It is of interest that TIMP-2 forms a complex with proMMP-2 through C-terminal domain of each molecule [10–13]. We, therefore, have examined whether the increased production of TIMP-2 by progesterone participated in the formation of the proMMP- 2-TIMP-2 complex by using gelatin–Sepharose. Most TIMP-2 found in the medium was recovered in the gelatin-binding fraction (Fig. 3B, lanes 1–4), and the distribution pattern of TIMP-2 was identical to that of proMMP-2 (Fig. 3A), indicating that most TIMP-2 in both media of the control and the progesterone-treated cells was complexed with proMMP-2.

Progesterone did not modulate the proMMP-2 production (Fig. 3A, lanes 1–4), whereas the amount of gelatin–Sepharose bound TIMP-2 increased in the progesterone-treated cells. These results suggested that the TIMP-2 increased by progesterone plays a significant role in proMMP-2 activation, since a complex formation of proMMP-2 with TIMP-2 prevents the proMMP-2 activation by *p*-aminophenylmercuric acetate (K. Imada, A. Ito, Y. Mori, Y. Itoh and H. Nagase, unpublished data) or by the cell surface activator [22]. Recently, Marbaix et al. [23] have reported that progesterone suppresses the release of active MMP- 2 from human endometrial explants. Although the mechanism was not investigated by these workers, the decreased release of active MMP-2 may be resulted from the increased production by progesterone of TIMP-2.

Uterine cervix is a typical connective tissue in which degradation of extracellular matrix occurs in a timely manner during cervical ripening and dilatation at term pregnancy. MMPs and TIMPs are considered to play a pivotal role in this process. In this regard, we previously reported that progesterone augmented the TIMP-1 production and effectively suppresses the MMPs production [9]. In this report, we have demonstrated that rabbit uterine cervical fibroblasts produce TIMP-2 in addition to TIMP-1, and that progesterone is a physiological stimulator of the production of both TIMP-1 and TIMP-2.

In conclusion, we suggest that progesterone plays an important role in maintaining the function of uterine cervix during pregnancy by overall suppression of matrix degradation by increasing the production of TIMP-2 which prevents the proMMP-2 activation along with the augmentation of TIMP-1 production and the suppression of proMMP-1 and proMMP-3 production.

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