Tissue Inhibitors of Matrix Metalloproteinases 1 and 2 and Matrix Metalloproteinase Activity in the Serum and Lungs of Mice with Lewis Lung Carcinoma

Ya. A. Kisarova and T. A. Korolenko

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 153, No. 6, pp. 856-860, June, 2012
Original article submitted February 24, 2011.

We studied the content of tissue inhibitors of matrix metalloproteinases 1 and 2 (TIMP-1 and TIMP-2) and activities of matrix metalloproteinases (MMP) in the serum and lungs of mice with Lewis lung carcinoma metastasizing into the lung. Metastasizing was associated with increased serum content of TIMP-1 and TIMP-2 (only on day 20 at the terminal stage of the tumor process). These data confirm the hypothesis on pro-tumorigenic role of TIMP-1 in the serum. Locally, the development of metastases was associated with a decrease in TIPM-1 concentration (day 7), an increase in TIMP-2 concentration (days 7 and 20), and elevated activity of MMP at all terms of the study (days 7, 15, and 20). Increased concentration of TIMP-2 in the lungs (but not in the serum) can be regarded as an indicator of Lewis lung carcinoma metastasizing.

Key Words: *TIMP-1; TIMP-2; MMP activity; Lewis lung carcinoma; metastases*

Matrix metalloproteinases (MMP), including gelatinases A (MMP-2) and B (MMP-9), play an important role in invasion and metastasizing of tumors by degrading collagens I-IV, laminin, fibronectin, elastin, and proteoglycans of basement membranes and other extracellular matrix components [3]. MMP-2 participates in the release of various growth factors (insulin-like growth factor, transforming growth factor β, *etc.*) promoting cell proliferation [3]. MPP are considered to be the targets of antitumor therapy, but this remains a little studied problem. In light of this, factors regulating MMP activity are of considerable interest. Tissue inhibitors of MMP, TIMP-1, TIMP-2, TIMP-3, and TIMP-4 are specific regulators of MMP activity [9]. TIMP can inhibit all types of MMP via binding to both active forms and proenzymes; however, considerable differences in specificity of their binding were noted [9]. The best studied representative of TIMP family, TIMP-1, binds to proMMP-9 and inactivates MMP-9 and to a lower extent other types of MMP (MMP-2, MMP-3, MMP-7) [3,13]. TIMP-1 is a bifunctional molecule; apart from inhibition of MMP-9, it exhibit pro-tumorigenic activity due to stimulation of the growth and inhibition of apoptosis of tumor cells and activation of angiogenesis [5]. TIMP-2 interacting primarily with membrane-type MMP (MT1-MMP, MMP-14) forms a complex MT1-MMP/TIMP-2 followed by activation of proMMP-2 and formation of active MMP-2 [6,8]. At the same time, TIMP-2 in high concentration inhibits a number of MMP, including MMP-2 and MMP-8 [3,14].

Biological functions of TIMP-1, TIMP-2, and various MMP are insufficiently studied and studies of tumor growth and metastasizing yields contradictory results. It was interesting to study the biological role of TIMP-1, TIMP-2, and MMP in experimental tumors, when the dynamics of the process can be traced, *i.e.* during tumor progression and metastasizing.

Here we evaluated the levels of TIMP-1 and TIMP-2 and compared them with changes in MMP ac-

Institute of Physiology, Siberian Division of the Russian Academy of Medical Sciences, Novosibirsk. ***Address for correspondence:*** y_kisarova@physiol.ru. Ya. A. Kisarova

tivities during the growth and metastasizing of Lewis lung carcinoma in mice.

MATERIALS AND METHODS

Experiments were carried out on C57Bl/6 male mice weighing 25-30 g (vivarium of the Institute of Physiology, Siberian Division of the Russian Academy of Medical Sciences) receiving transplantation of Lewis lung carcinoma (LLC) metastasizing into the lung. Tumor cells were transplanted into the thigh muscles ($0.5\text{-}1.0\times10^6$ per mouse). The animals were decapitated on days 7 (start of metastasizing), 15 (progression of the tumor process), and 20 (maximum development of the tumor and metastases). All procedures during homogenate preparation were carried out on cold (0-4°C) [2]. Blood serum was isolated by centrifugation at 3000*g* and 4°C for 20 min (Eppendorf 5415 R centrifuge).

The concentration of TIMP-1 and TIMP-2 in the mouse lung tissue and blood serum was quantitatively measured by ELISA using appropriate commercial kits for mice (Ray Biotech Inc.). Antibodies specific to TIMP-1 and TIMP-2 were added to 96-well plates. Primary biotinylated antibodies and secondary streptavidin-conjugated antibodies labeled with horseradish peroxidase were used. No cross-reaction with cytokines and adhesion molecules were noted. The minimum measured concentrations of TIMP-1 and TIMP-2 were 3 pg/ml and <80 pg/ml, respectively. Extinction of samples was evaluated using a Stat Fax-2100 microplate reader (Awareness Technology Inc.). For measuring the concentration of TIMP-1 and TIMP-2, the samples were standardized by cell protein content. The results were expressed in ng/ml serum or ng/mg protein in the lung tissue.

Activity of MMP was measured fluorometrically against substrate MCA-Pro-Leu-Gly~Leu-Dpa-Ala-Arg-NH_2 (American Peptide Company, Inc.) at pH 7.5, where MCA is 7-methoxycoumarin-4-acetyl, Dpa is N-3-(2,4-dinitrophenyl)-L-α,β-diaminopropionyl, a fluorescence quencher [7] that allowed measuring activities of primarily MMP-2 and MMP-7 (matrilysin) cleaving Gly~Leu bonds [7]. According to modern proteomic analysis data, activity of other MMP (MMP-1, MMP-3, MMP-8) can also be measured [4]. Fluorescence was measured using a Shimadzu RF-5301 PC fluorometer at 328 nm (excitation) and 393 nm (emission). 7-Methylcoumarin (ICN Biomedicals Inc.) was used as the standard. Incubation was performed in the presence of specific serine protease inhibitor phenylmethylsulfonyl fluoride (Boehringer Mannhein) in a concentration of 0.5 mM. The results were expressed in μmol MCA/liter for blood serum and μmol MCA/g protein for lung homogenates.

The data were processed statistically using Statistica 6.0 software. The data distribution was evaluated using Kolmogorov-Smirnov test and significance of differences was assessed by one-way ANOVA and Student *t* test. The significance level α was taken as 0.05.

RESULTS

We have previously demonstrated that the formation of LLC is associated with increased concentrations of TIMP-1 in mouse serum, which attests to pronounced shifts in MMP/TIMP-1 ratio [1,2]. In the present study, the serum TIMP-1 content in tumor-bearing mice at all terms after tumor transplantation was higher than in intact animals (Fig. 1, *a*). Changes in serum TIMP-2 concentration differed from those observed for TIMP-1 (Fig. 1, *b*). In intact mice, the concentration of TIMP-2

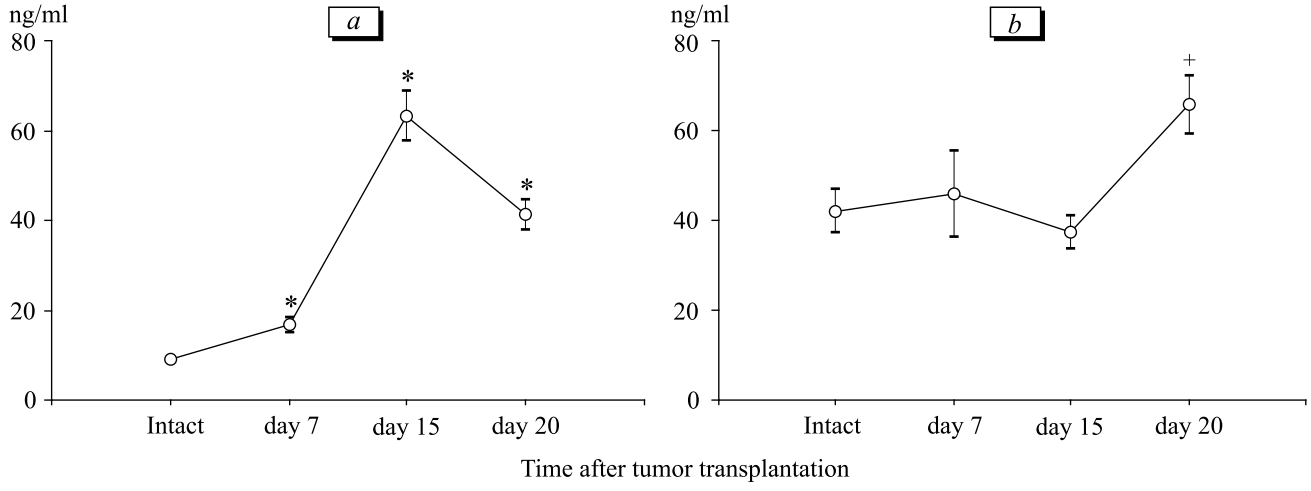

Fig. 1. Concentrations of TIMP-1 (*a*) and TIMP-2 (*b*) in mouse serum in the dynamics of LLC development (*M*±*m*). $p<0.05$ in comparison with: *intact mice, +intact mice and mice with LLC on days 7 and 15.

almost 4-fold surpassed that of TIMP-1 (Fig. 1, *a*, *b*), while in tumor-bearing mice the levels of TIMP-2 and TIMP-1 differed by 1.5-2.0 times. The level of TIMP-2 was elevated only at the terminal stage of the tumor process (day 20) in comparison with the corresponding parameter in intact animals and mice with LLC at earlier terms (days 7 and 15; Fig. 1, *b*).

In the lung tissue, TIMP-1 concentration in mice with LLC (day 7) was lower that in intact mice (Fig. 2, *a*). The content of TIMP-2 in the lung tissue on days 7 and 20 of the tumor process was higher than in intact mice (Fig. 2, *b*). In mice with LLC, activity of MMP in the lung tissue was higher than in intact animals at all terms of the study (days 7, 15, and 20; Fig 3, *a*). These changes in MMP activity were probably typical of tumor metastasizing into the lung.

Serum MMP activity increased on day 7, decreased on day 15 (metastasizing and LLC progression), and increased again on day 20 (Fig. 3, *b*).

Thus, the most important findings were considerable elevation of serum TIMP-1 concentration at all terms of tumor growth and increase in TIMP-2 only on day 20, *i.e.* at the terminal stage of the tumor process (which attest to different induction of endogenous MMP inhibitors and pro-tumorigenic activity of TIMP-1), high local MMP activity in the lungs with tumor metastases, and opposite changes in TIMP-1 and TIMP-2 (decrease in TIMP-1 concentration on day 7 and increase in TIMP-2 content on days 7 and 20). Judging from progressively increasing MMP activity, the parameters in the lungs with metastases differ from the corresponding changes in the LLC tumor tissue (increase in MMP activity due to predominance of inactive MMP forms during tumor development) [1].

Comparison of serum TIMP-1 and TIMP-2 content showed that the increase in TIMP-1 concentration is the most reliable and earlier sign that can serve as a predictor of LLC development and metastasizing in mice. Normally, TIMP-1 is present in mammalian serum in low concentrations, but some tumors in humans (e.g. colorectal cancer) are associated with the appearance of aberrant glycosylated TIMP-1, which has a diagnostic value [13]. In mice with LLC, the increase in serum TIMP-2 concentration lagged behind TIMP-1 rise, which reduced its prognostic value. Locally, different changes in TIMP-1 and TIMP-2 concentrations were noted during tumor metastasizing (Fig. 2, *a*, *b*). TIMP-2 is less studied than TIMP-1: this is a non-glycosylated protein with a molecular weight of 21 kDa forming a complex with pro-enzyme MMP-2, while TIMP-1 is a glycosylated 29-kDa protein forming a complex with pro-MMP-9 [12]. The role of TIMP-2 in tumors is related to activation of angiogenesis and inhibition of tumor invasion. However, this is observed only in some types of tumors. No decrease in TIMP-2 in the tumor was shown at the level of transcription. It should be noted that tumor growth is accompanied by the formation of tumor-associated stromal cells and extracellular matrix affecting the tumor tissue and promoting its growth. According to our findings, the increase in TIMP-2 level in the lungs is related to the formation of lung metastases.

It is known that TIMP stoichiometrically bind to MMP at 1:1 ratio. Normally, the concentrations of endogenous MMP inhibitors TIMP in tissues and extracellular fluids surpass the level of MMP, which prevents MMP-induced proteolysis [11]. TIMP, similarly to MMP, are primarily produced by stromal cells. TIMP transcription is regulated by factors similar to the factors regulating MMP activity (TNF-α, IL-1, IL-6, *etc.*) [12]. TIMP-1, similarly to TIMP-3 and TIMP-4, is a highly inducible protein at the level of transcription. TIMP-1 expression is activated by some cytokines, tumor promoters, oncoproteins, and some

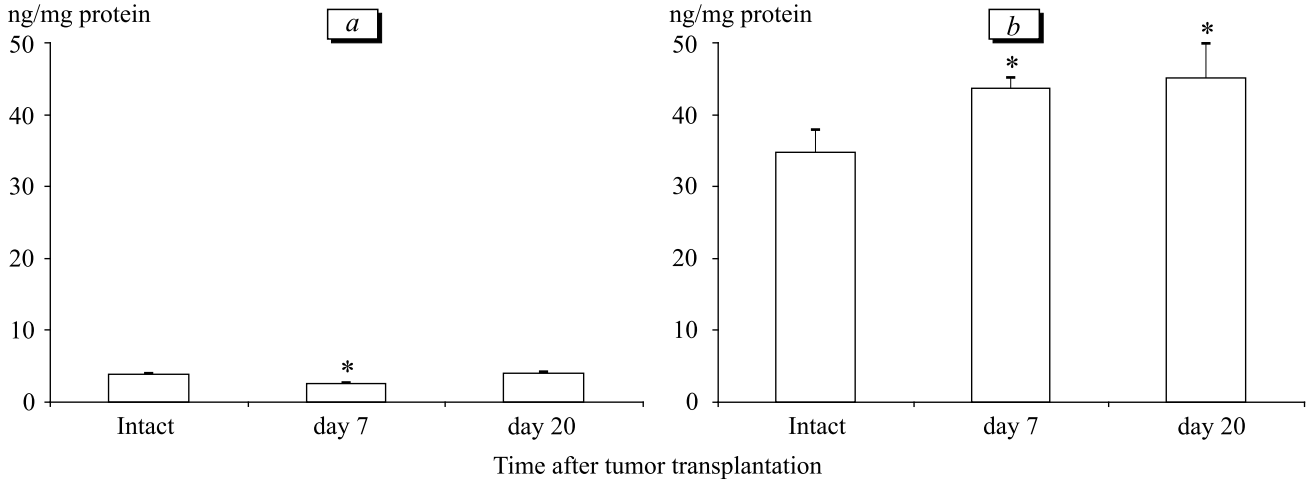

Fig. 2. Concentrations of TIMP-1 (*a*) and TIMP-2 (*b*) in the lungs of mice in the dynamics of LLC development (*M*±*m*). **p*<0.05 compared to intact mice.

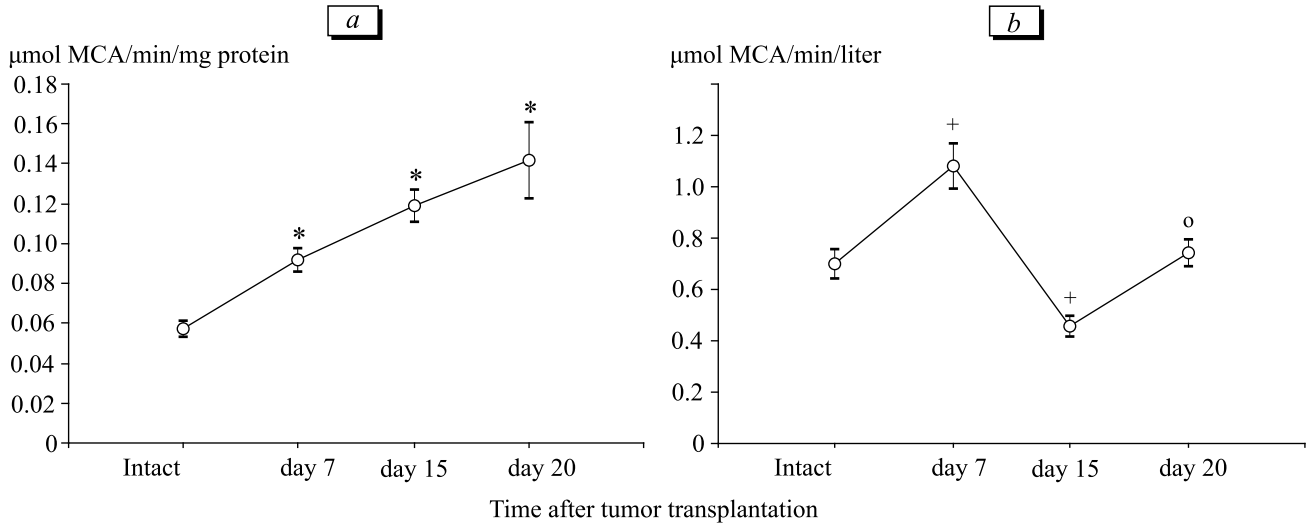

Fig. 3. Activity of MMP in lung homogenates (*a*) and blood serum (*b*) of mice with LLC (*M*±*m*). $p<0.05$ in comparison with: *lungs of intact mice, +serum of intact mice; °serum of mice on days 7 and 15 of LLC development.

mitogenic factors [15]. In the studied LLC model, the increase in blood TIMP-1 level is related to tumor growth and the influence of the above-specified growth factors. The observed increase in the concentration of TIMP-1 (and partially TIMP-2) in the serum can be determined by their enhanced secretion by stromal cells (fibroblasts), probably, under the effect of EMMPRIN, which requires further investigations. Under physiological *in vivo* conditions, MMP are expressed in low amounts and are controlled by various factors, including IL-1, IL-4, IL-6, transforming growth factors (EGF, HGF, TGF-β), TNF-α [10]. These factors and cytokines in turn can be activated by MMP by the feedback mechanism. An opposite effect of TGF-β on MMP and TIMP-1 was reported: suppression of MMP expression and simultaneous activation of TIMP-1 transcription [15]. The increase in MMP activity in the lungs with metastases is evidently induced by these factors. MMP plays a more complex role during tumor growth than was previously thought. They not only participate in degradation of extracellular matrix components during invasion and metastasizing, but also are involved into the early stage of tumorigenesis associated with angiogenesis, cell migration, and production of new types of MMP by tumor cells [10]. It is evident that MMP and TIMP can be targets for various drugs during antitumor therapy, but this approach is not always effective *in vivo* and requires more detailed investigations.

REFERENCES

1. Ya. A. Kisarova, *Byull. Sib. Otd. Ross. Akad. Med. Nauk*., No. 2, 101-108 (2011).
2. T. A. Korolenko, T. G. Filatova, V. M. Belichenko, *et al., Byull. Eksp. Biol. Med*., **150**, No. 8, 198-201 (2010).
3. M. Bogusiewicz, M. Stryjecka-Zimmer, K. Postawski, *et al. Gynecol. Endocrinol.,* **23**, No. 9, 541-546 (2007).
4. G. B. Fields, *Methods Mol. Biol*., **622**, 393-433 (2010).
5. H. Ishida, N. Murata, Y. Hayashi, *et al., Surg. Today*., **33**, No. 12, 885-892 (2003).
6. A. Jezierska and T. Motyl, *Med. Sci. Monit*., **15**, No. 2, 32-40 (2009).
7. C. G. Knight, F. Willenbrock, and G. Murphy, *FEBS Lett*., **296**, No. 3, 263-266 (1992).
8. M. A. Lafleur, M. M. Handsley, and D. R. Edwards, *Expert Rev. Mol. Med*., **5**, No. 23, 1-39 (2003).
9. E. Morgunova, A. Tuuttila, U. Bergmann, and K. Truggvason, *Proc. Natl. Acad. Sci. USA*., **99**, No. 11, 7414-7419 (2002).
10. C. J. Morrison, G. S. Butler, D. Rodriguez, *et al*., *Cur. Opin. Cell. Biol.*, **21**, No. 5, 645-653 (2009).
11. L. Nakopoulou, I. Tsirmpa, P. Alexandrou, *et al., Breast Cancer Res. Treat*. **77**, No. 2, 145-155 (2003).
12. Q. H. Nie, C. L. Zhu, Y. F. Zhang, *et al., Dig. Dis. Sci.*, **55**, No. 5, 1286-1295 (2010).
13. W. G. Stetler-Stevenson, *Sci. Signal.,* **1**, No. 27, re6 (2008).
14. W. G. Stetler-Stevenson and D. W. Seo, *Trends Mol. Med.*, **11**, No. 3, 97-103 (2005).
15. A. Zalatnai, *Cancer Microenvironment and Therapeutic Implications*. **2**, 19-38 (2009).