IMMUNOLOGY AND MICROBIOLOGY

Sorsby Fundus Dystrophy-Related Mutation in Tissue Inhibitor of Metalloproteinases-3 Impairs Regulation of Its Expression in Mouse Fibroblasts

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 143, No. 1, pp. 70-73, January, 2007
Original article submitted July 13, 2006

Mutations in the C-terminal domain of tissue inhibitor of metalloproteinases-3 (TIMP-3) are linked with Sorsby fundus dystrophy (autosomal dominant retinal dystrophy). Experiments on *timp-3* gene knock-out and knock-in mice, carriers of dystrophy-linked Timp-3^{S156C} mutation, showed that expression of mutant Timp-3^{S156C}, in contrast to the normal one, is not regulated by phorbol-myristate-12-acetate and is retained at a high level in a medium without FCS. The mutant protein is not transported into the nucleus, while fibroblasts expressing it survive without FCS and other growth factors. It is hypothesized that normal Timp-3 is involved in the regulation of expression of its own *timp-3* gene and, probably, other genes associated with growth and proliferation. The absence of this characteristic in mutant Timp-3^{S156C} is presumably responsible for its accumulation in the extracellular matrix.

Key Words: *Sorsby fundus dystrophy; tissue inhibitor of metalloproteinases-3*

Tissue inhibitor of metalloproteinases-3 (TIMP-3) is a component of extracellular matrix (ECM). This polyfunctional protein not only inhibits activities of some matrix metalloproteinases, but also participates in cell transformation, migration, and invasion processes, apoptosis and angiogenesis [1,13]. The expression of TIMP-3 is detected in all types of tissues and cells, is related to cell cycle, and is regulated by steroid hormones, growth factors, and cytokines [2,7,12]. The Ser156Cys mutation in the TIMP-3 C-terminal domain is associated with the development of one of hereditary forms of macular dystrophy, Sorsby dystrophy [11]. Clinical manifestations of this rare autosomal dominant disease resemble symptoms of age-associated macular degeneration, one of the most frequent causes of blindness in elderly people [10].

To study the molecular mechanisms underlying Sorsby fundus dystrophy transgenic knock-out mice lacking *timp-3* expression and knock-in mice, carriers of orthologous Ser156Cys mutation in the corresponding murine gene were generated [9,11]. Human TIMP-3^{S156C} and murine Timp-3^{S156C} have similar biochemical properties [11], are accumulated in the retinal pigmented epithelium of patients with Sorsby fundus dystrophy [3,4] and knock-in mice [11], as well as in ECM of fibroblast culture from these animals [9]. Therefore, the mice carrying Timp-3^{S156C} mutation are used as the biological model of Sorsby fundus dystrophy, while fibroblast cultures from these animals are used for studies of Timp-3^{S156C} characteristics.

Since accumulation of TIMP-3 in ECM can be a direct cause of Sorsby fundus dystrophy [6], we studied the mechanisms of this process.

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MATERIALS AND METHODS

Cell cultures of normal mouse fibroblasts and fibroblasts from mice homozygous for Timp-3^{S156C} mutation [9] were used in the study. The cells were cultured in DMEM with 10% FCS. In order to obtain ECM, the cells were cultured until confluence and treated with PBS containing 5 mM EGTA and 1 mM PMSF for 15 min at 37°C [9]. Cell removal was verified by microscopy. ECM after cell removal was dissolved in a small volume of Laemmli buffer [11] and used in subsequent experiments. Protein content in ECM extract was evaluated using Bradford's reagent (BioRad).

Samples of ECM calibrated for protein content were applied onto a 12.5% PAAG and electrophoretically separated under reducing conditions. After electrophoresis the proteins were transferred onto Immobilon polyvinylidenefluoride membrane (Millipore). Immunodetection of Timp-3 was carried out using specific rabbit antibodies to a peptide corresponding to amino acids 196-211 of murine Timp-3 [11]; laminin was detected using monoclonal antibodies to mouse laminin B2 chain (Upstate Biotechnology). Peroxidase-labeled antibodies to rabbit or mouse IgG (Calbiochem) served as second antibodies. The signal was detected using ECL reagent (Amersham).

The cells were grown in DMEM containing 10% FCS until 30% confluence in Nunc chambers. Nutrient medium was removed, the cells were washed with PBS, fixed in 100% acetone at 4°C, and dried. Before staining the cells were washed in a buffer containing 0.1% Twin-200 (Sigma) and incubated in the presence of 5% BSA (Sigma) and 0.1% Twin-200 at 20°C for 1 h. Rabbit antibodies to Timp-3 peptide diluted 1:100 were used for staining. The antibodies were dissolved in PBS with 0.1% Twin-200 and applied onto prepared cells. The samples were incubated for 1 h at 20°C, washed 3×10 min in a buffer with 0.1% Twin-200. Goat antibodies to light and heavy chains of rabbit IgG labeled with Alexa Fluor fluorescent dye (Molecular Probes) in 1:1000 dilution served as second antibodies. Second antibodies were diluted in a buffer with 0.1% Twin-200 and applied onto the samples. After 1-h incubation at ambient temperature stained cells were washed 3×10 min in a buffer with 0.1% Twin-200 and examined under a microscope.

Experiments on cell survival were carried out as follows. The cells were cultured in DMEM with 10% FCS until confluence, treated with trypsin, washed in sterile PBS, and centrifuged. Cell suspensions were inoculated in dishes (10^6 cells/cm^2) and cultured in DMEM without FCS until the end of the experiment. On days 3 and 4 the cells (3 dishes of each cell culture) were harvested with trypsin, washed, centrifuged, and the percent of living cells in each dish was evaluated daily (from the number of cells at the start of incubation). The mean of 3 measurements was used in the diagram.

RESULTS

The content of normal Timp-3 protein in cultured fibroblast ECM without mitogenic stimulation (Fig. 1) was significantly lower than in the presence of phorbol-myristate-12-acetate (PMA). The content of Timp-3^{S156C} in ECM of fibroblasts homozygous for Ser156Cys mutation was high and did not depend on mitogenic stimulation. The expression of mutant Timp-3^{S156C} seems to be constitutive. The presence of dexamethasone in culture medium suppressed PMA-induced activation of Timp-3 expression in normal fibroblasts and did not modify the synthesis of mutant Timp-3^{S156C} (data not presented).

Normal Timp-3 protein in fibroblasts at 30% confluence was located in the cytoplasm and nucleus (Fig. 2). The cytoplasm was evenly stained, while in the nucleus more intensely stained conglomerates were seen. By contrast, Timp-3^{S156C} virtually did not stain the nucleus, while its distribution in the cytoplasm formed a reticular structure with alternating more and less intensively stained areas (Fig. 2, *a*). The shape of fibroblast expressing the mutant Timp-3^{S156C} differed from the normal (a large flat cell with a large nucleus).

Normal fibroblasts cultured without growth factors virtually completely died by day 4 of the experiment, while homozygous carriers of Timp-3^{S156C} mutation survived (Fig. 3).

Dysregulation of mutant Timp-3^{S156C} expression paralleled by the loss of sensitivity of cultured fibroblasts to mitogen (PMA) and differentiation stimuli (dexamethasone) suggests that normal Timp-

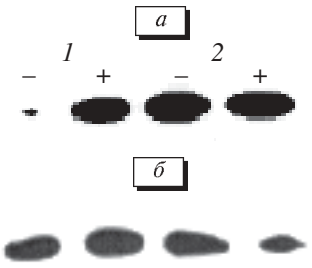

Fig. 1. Expression of Timp-3 protein (*a*) and laminin (*b*) in ECM of mouse fibroblasts cultures in serum-free DMEM in the absence (–) and presence (+) of PMA. *1*) normal fibroblasts; *2*) fibroblasts from mice homozygous for Ser156Cys mutation. Staining with antibodies to Timp-3 and laminin, respectively.

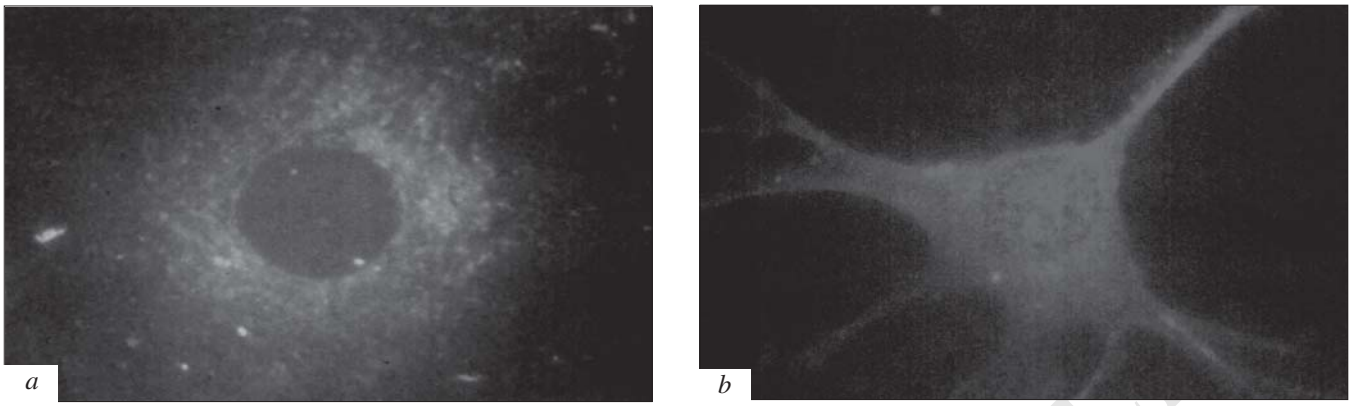

Fig. 2. Intracellular distribution of mutant (*a*) and normal (*b*) Timp-3 in mouse fibroblasts reaching 30% confluence. Unstained nucleus and more intensive specific signal from the cytoplasm of homozygous mutant fibroblasts in comparison with normal cells. The shape of mutant cells, differing from normal, is well discernible. Staining with Timp-3 specific antibodies, ×400.

3 protein is directly involved in the regulation of its own expression by the negative feedback mechanism and/or mutant Timp-3^{S156C} is a potent mitogen.

The presence of sufficient amount of normal Timp-3 in the EMC seems to signal the cell to stop the synthesis of this protein. Normal protein is absent in the ECM of fibroblasts expressing exclusively mutant Timp-3^{S156C}, hence, the feedback mechanism cannot be triggered and the cells produce mutant protein, which is accumulated in the ECM. Presumably, the same mechanism underlies accumulation of mutant TIMP-3 in the Bruch's membrane of patients with Sorsby fundus dystrophy.

The proliferation of fibroblasts (homozygous carriers of Timp-3^{S156C} mutation) in the absence of growth factors is comparable with normal cell multiplication in the presence of growth factors. Presumably, this is due to the fact that normal Timp-3 interacts with PMA-sensitive elements not only at its own promotor, but also on promotors of other genes, responsible for cell growth and multiplication. In this case Timp-3 protein should penetrate into the nucleus. The results of immunocytochemical studies indicate that normal Timp-3, in contrast to the mutant protein, is located not only in the cytoplasm, but also in the nucleus, which confirms the previous hypothesis. Striking differences in the morphological and physiological phenotypes of mutant and normal fibroblasts [9] can be explained by the regulatory effect of normal Timp-3 on genes responsible for the corresponding signs. Hence, we note for the first time that Timp-3 protein seems to possess characteristics of a transcription factor. The loss of this heretofore not described function is associated with accumulation of mutant Timp-3^{S156C} in the ECM and can be a component in the pathogenesis of Sorsby fundus dystrophy.

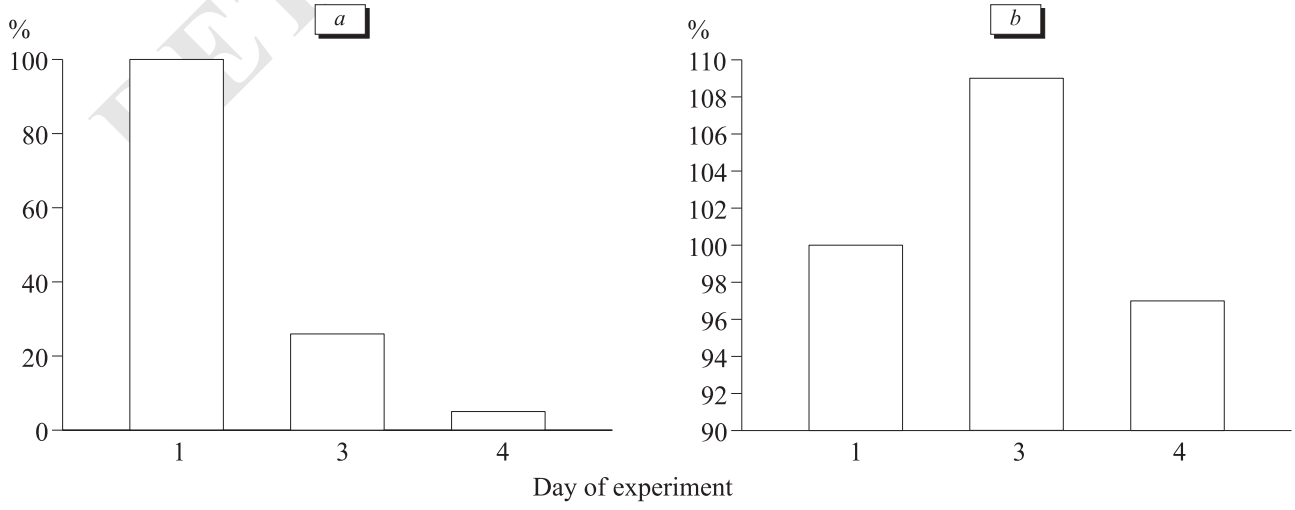

Fig. 3. Survival of normal mouse fibroblasts (*a*) and fibroblasts homozygous for Timp-3^{S156C} mutation (*b*) in serum-free DMEM.

If mutant Timp-3^{S156C} is characterized by its own mitogenic activity, its effect on cells resembles the stimulatory effect of PMA. The potentiating effects of TIMP-1 and TIMP-2 on different cell types are well known [5,8]; chicken homologue ChIMP-3 activates the growth of nontransformed fibroblasts under conditions of low (0.1%) serum content [14]. Normal Timp-3 is expressed in response to mitogenic stimuli, while mutant Timp-3^{S156C} itself presumably possesses mitogenic characteristics. More detailed study of these proteins will be the object of further investigation.

The study was partially supported by German Research Society (grant No. SFB518/TP13).

The authors are grateful to Prof. B. Weber (University of Wuerzburg) for granting the possibility of working with animals and cell cultures and for financial, organization, and other support.

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