Brief Report

Expression of TIMP-3 Gene by Construction of a Eukaryotic Cell Expression Vector and Its Role in Reduction of Metastasis in a Human Breast Cancer Cell Line

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The present study is aimed at studying the gene for TIMP-3, a mammalian tissue inhibitor, by constructing a recombinant eukaryotic cell vector for gene therapy in human breast cancer. We obtained the TIMP-3 gene from the human placent by RT-PCR. TIMP-3 gene was subcloned into pcDNA3.1 vetor from pMD18T vector by means of gene cloning to construct pcDNA3.1 recombinant vector. Human breast cancer cell line MDA-MB-453 was transfected with pcDNA3.1-TIMP3 recombinant vector using lipofectamine reagent. Then the expression of TIMP-3 and the effect on the metastasis of MDA-MB-453 were examined. The correct construction of pcDNA-TIMP3 was identified by means of restriction enzyme analysis, PCR amplication and nucleotide sequencing. Western blotting showed that the transfected cells were able to express TIMP-3, indicating that our construction of the pcDNA-TIMP3 eukaryotic expression vector was constructed successfully. Our experiments further indicated that the potential of metastasis was significantly reduced for the transfected cell line MDA-MB-453. *Cellular & Molecular Immunology*. 2004;1(4):308-310.

Key Words: TIMP-3 gene, eukaryotic expression, DNA recombinant technology, transfection

Introduction

The matrix metalloproteinase (MMPs) were a family of zinc-containing protease, which collectively are capable of degrading all components of the extracellular matrix (ECM) (1). In particular, they were the only enzymes known to degrade fibrillar collagen (2). There were currently at least 28 human MMPs reported. Degradation of the ECM was an important feature of tumor invasion and metastasis and it was now widely recognized that the MMPs had a major role in these processes. The activity of MMPs was regulated at several levels, including gene transcription, activation of secreted proenzymes, and inhibition by a class of natural inhibitors called TIMPs (tissue inhibitors of metalloproteinases). TIMPs were secreted proteins that inhibit MMPs by binding to their active site in a 1:1 ratio. Recently, two new members of the TIMP family have been cloned: TIMP-3 and TIMP-4. Of the four known TIMPs, TIMP-3 was distinguished by its tighter binding to the extracellular matrix. TIMP-3 binding may be important for the cellular regulation of activity of the MMPs. To explore the physiological role of TIMP-3 in human breast cancer at gene levels and study the application of TIMP-3 gene therapy in breast cancer, we have constructed a mammalian TIMP-3 recombinant eukaryotic cell vector.

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Received for publication Jun 14, 2004. Accepted for publication Aug 16, 2004.

Materials and Methods

Materials

Eukaryotic expression vector-pcDNA3.1 was the product of Vector Co., USA; pMD18-T TA vector was the product of TaKaLa Co., Japan; Taq DNA polymerase and restriction endonuclease (XbaI, EcoRI) were the products of Promerga Co., USA; breast cancer cell line MDA-MB-453 was purchased from Shanghai cell biochemical institute, China Academy of Science; Lipofectimine was the product of GIBCO BRL Co., USA. Human breast cancer cells MDA-MB-453 were cultured in Iscove's modified medium (IMDM) containing 10% fetal bovine serum (FCS), 100 units/ml penicillin, and 100μg/ml streptomycin and maintained in a humidified atmosphere at 37 °C in 5% carbon dioxide.

Construction of TIMP-3 eukaryotic expression vector

We extracted TIMP-3 mRNA from human fresh placent using the method of fluorescein isothiocyanate. Then we obtained the full length TIMP-3 cDNA by RT-PCR. Sense primer: 5'-AAG AAT TCA TGA CGC CTT GGC TCG GG-3'; anti-sense primer: 5'-GGT CTA GAT CAG GGG TCT GTG GCA TT-3'. We inserted the TIMP-3 cDNA to the vector pMD18-T. After sequencing the TIMP3-

Abbreviations: TIMP, tissue inhibitor of metalloproteinase; MMPs, matrix metalloproteinase; ECM, extracellular matrix.

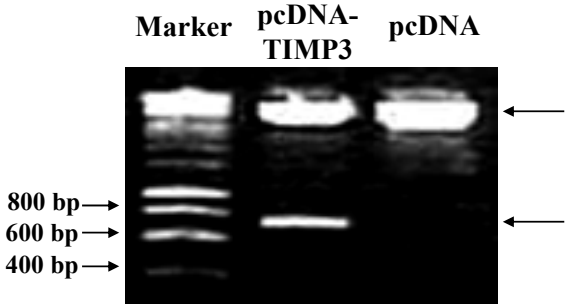

Figure 1. The recombinant pcDNA-TIMP3 was digested with EcoR I and Xba I. The vector pcDNA3.1 and the fragment of TIMP3 were indicated by an arrow.

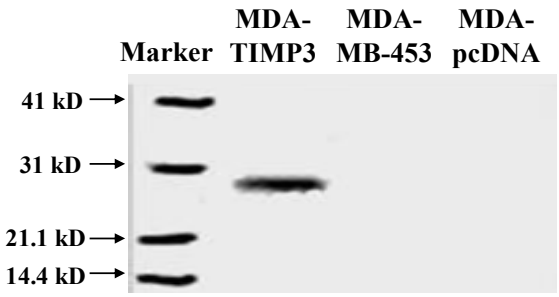

Figure 2. Western-blotting identified TIMP-3 recombinant protein. Cells transfected with MDA-TIMP3 expressed the protein TIMP-3, but MDA-pcDNA and MDA-MB-453 did not.

pMD18-T vector and pcDNA3.1 were digested with EcoR I and Xba I. The fragment from TIMP3-pMD18-T was inserted into the pcDNA3.1. The recombinant vector was transformated into colibacillus JM109. After amplification the recombinant vector was extracted and identified using EcoR I and Xba I digesting.

Gene transfection

The recombinant pcDNA-TIMP3 was transfected into the MDA-MB-453 by lipofectimine kit according to the manufacturer's instructions. As control the vector pcDNA3.1 was transfected into the MDA-MB-453.

Detection of cell proliferation

By MTT reduction assay, proliferation curve of the cells was obtained according to the value of OD.

In Vitro Invasion Assay

Boyden chamber was used to estimate the invasive ability of transfected MDA-MB-453 cells as described by Baker (1).

Results

We obtained the full length cDNA of TIMP-3 from placent. And the cDNA was completely correct by sequencing. When the recombined vector pcDNA-TIMP3 was digested by EcoR I and Xba I, one segment about 633 bp was observed by agarose electrophoresis (Figure 1).

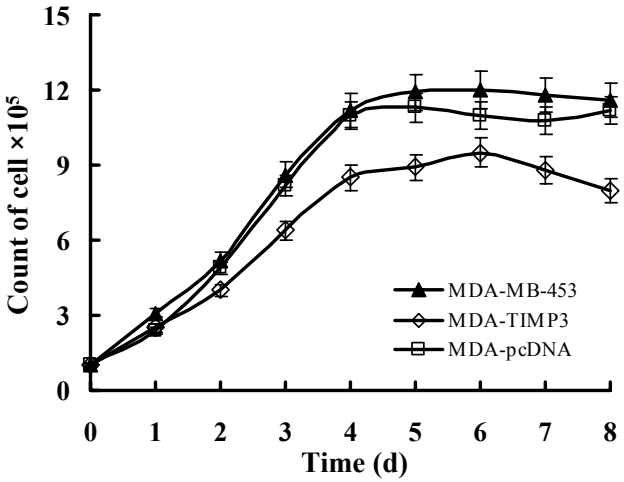

Figure3. Cell growth curve of cell MDA-MB-453, MDA-TIMP3 and MDA-pcDNA. Growth velocity of cell MDA-TIMP3 were significantly slower than cell MDA-pcDNA and MDA-MB-453 ($p<0.05$).

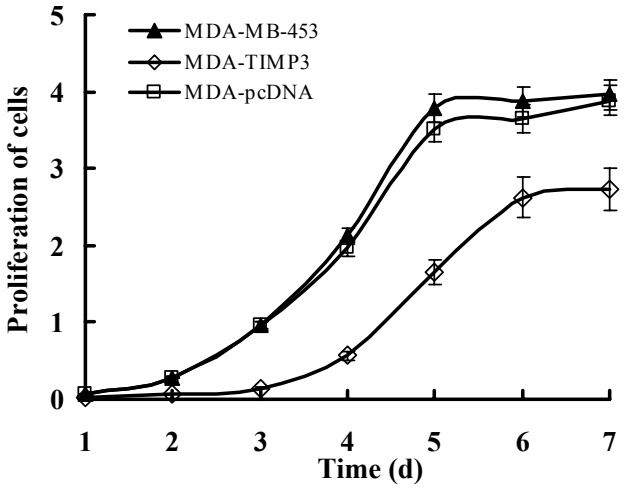

Figure 4. Proliferation curve of cell MDA-MB-453, MDA-TIMP3 and MDA-pcDNA. The ability of cell proliferation of cell MDA-TIMP3 were weaker than cell MDA-MB-453 and MDA-pcDNA by means of OD value ($p<0.05$).

After the pcDNA-TIMP3 and pcDNA3.1 were transfected into the cell MDA-MB-453, we used G418 in concentration of 800 μg/ml to select the resistant cell clone for two weeks and then 400 μg/ml for two weeks. While the control group-MDA-MB-453 was killed by G418 several resistant cell clones in the group transfected with pcDNA-TIMP3 and pcDNA3.1 survived and we name them cell MDA-TIMP3 and cell MDA-pcDNA. It was proved by Western blotting that cell MDA-TIMP3 expressed the protein TIMP-3 while cell MDA-pcDNA and MDA-MB-453 did not (Figure 2).

The cell MDA-TIMP3 and cell MDA-pcDNA's shape,

volume and ability of anchoring growth were not different compared with MDA-MB-453.

Growth velocity of cell MDA-TIMP3 was significantly slower than cell MDA-pcDNA and MDA-MB-453 ($p<0.05$). The ability of cell proliferation of cell MDA-TIMP3 was weaken than cell MDA-MB-453 and MDA -pcDNA by means of the mean of MTT(Figure3, 4).

In the Boyden chamber assay, the amount of cell MDA-TIMP3 which penetrated reconstituted basement membrane was 21.3±2.5 and it was significantly lower than the amount of cell MDA-MB-453 81.7±4.6 and MDA-pcDNA 77.5±6.4 ($p<0.05$).

Discussion

The MMP are a family of structurally related neutral proteinases involved in the remodeling of ECM in processes such as fetal development, wound healing, inflammation, and tumor invasion (1). These enzymes have overlapping specificities, being able to degrade important ECM macromolecules such as different types of collagens, laminin, proteoglycans, elastin, and fibronectin (2). The activity of metalloproteinases is regulated at several levels, including gene transcription, activation of secreted proenzymes, and inhibition by a class of natural inhibitors called TIMPs. TIMPs are secreted proteins that inhibit MMPs by binding to their active site in a 1:1 stoichiometric ratio. MMPs and their inhibitors TIMPs play a critical role in ECM homeostasis. Controlled remodeling of the ECM is an essential aspect of normal development, and deregulated remodeling has been indicated to have a role in the etiology of diseases such as arthritis, periodontal disease, and cancer metastasis (3). Four mammalian TIMPs have been identified so far: TIMP-1, TIMP-2 and the recently cloned TIMP-3, TIMP-4 (4). The proteins are classified based on structural similarity to each other, as well as their ability to inhibit metalloproteinases.

In our search active recombinant TIMP-3 protein is required for characterization of its biochemical activity against MMPs and biological functions in inhibiting tumor growth and metastasis. Proteins of eukaryotic cells expressed in *Escherichia coli* are often generated as inactive, insoluble aggregates known as inclusion bodies and therefore require *in vitro* complicated refolding. In the present study, we constructed the eukaryotic vector of TIMP-3 and named pcDNA-TIMP3.Then we transfected it into the cell line MDA-MB-453. The cell MDA-TIMP3 expressed the 28 kD TIMP3 protein by Western-blotting identification. So we can conveniently observe the effect of TIMP-3 protein on invasion of breast cancer cell.

In this study we also demonstrated an inhibitory effect of cell invasion on the TIMP-3-transfected cells compared with the TIMP-3 negative control cells. In the experimental Boyden invasion assay, significantly inhibition of invasion potential was achieved when the breast cancer cells were transfected with pcDNA-TIMP3. The obviously suppression of invasion potential of breast cancer cells by TIMP-3 suggested that the major matrix degradation proteinases required for the invasion of breast cancer cells in the Matrigel invasion assay are MMPs, and their enzymatic activities can be inhibited effectively by TIMP-3. The inhibition of breast cancer cell invasion by both an exogenous supply of rTIMP3p and the endogenous expressed TIMP-3 suggested that the TIMP-3-mediated anti-invasion activity could be physiologically or pathologically relevant in the tumor microenvironment.

In this study, we observed that the growth and proliferation potential of the breast cancer cells transfected with TIMP-3 was obviously inhibited. We thought the possible mechanism was that a major consequence of ECM degradation by MMPs was the release of ECM-sequestered growth factors, several of which played an important role in tumor cell survival, and proliferation as well as angiogenesis (5). Some of these growth factors, including transforming growth factor (TGF), insulin-like growth factors (IGF-I and II) and basic fibroblast growth factor (bFGF) are maintained in latent form complexed to various binding proteins (6).

The down-regulation of MMPs may occur at the levels of transcriptional regulation of the genes, activation of secreted proenzymes, and through interaction with TIMPs. The clinical importance of MMPs during the tumor progression emphasizes the need to effectively block MMPs and the subsequent tumor cell invasion. The inhibitory effect of TIMPs on MMP activity leads one to expect that an increase in the amount of TIMPs relative to MMPs could function to block tumor cell invasion and metastasis. This study suggested that an inhibitory activity of TIMPs played an important role in inhibiting tumor cell malignant progression leading to invasion and metastasis. Therefore, the potential therapeutic value of TIMP-3 for controlling cancer progression warrants further investigation.

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