Overexpression of Tissue Inhibitor of Metalloproteinases-2 by Retroviral-mediated Gene Transfer in Vivo Inhibits Tumor Growth and Invasion

Suzan Imren, Donald B. Kohn, Hiroyuki Shimada, Laurence Blavier, and Yves A. DeClerck

Division of Hematology-Oncology and Division of Research Immunology and Bone Marrow Transplantation, Departments of Pediatrics [S. I., D. B. K., L. B., Y. A. D.], Biochemistry and Molecular Biology [Y. A. D.], Molecular Microbiology and Immunology [D. B. K.], and Pathology [H. S.], Childrens Hospital Los Angeles and University of Southern California, Los Angeles, California 90027

Abstract

We have demonstrated previously that overexpression of tissue inhibitor of metalloproteinases-2 (TIMP-2), an inhibitor of matrix-degrading metalloproteinases, not only inhibits the invasive and metastatic behavior of tumor cells but also significantly decreases tumor growth in vivo (Y. A. DeClerck et al., Cancer Res., 52: 701–708, 1992). This latter effect was found to be dependent on the ability of TIMP-2 to prevent the degradation of the collagen matrix (A. M. Montgomery et al., Cancer Res., 54: 5467–5473, 1994). In this report, we have overexpressed TIMP-2 in tumor tissue by retroviral-mediated gene transfer into tumor cells by co-injecting s.c. in nude mice tumorigenic c-Ha-ras-transfected rat embryo fibroblasts with irradiated packaging cells producing high titer retroviral vectors containing the human TIMP-2 cDNA. The growth rate of tumors derived from cells co-injected with the TIMP-2 vector producer cells was significantly slower than the growth rate of tumors derived from cells co-injected with packaging cells producing a retrovirus containing the Escherichia coli β-galactosidase gene. The transduction efficiency was estimated at 13%, and the production of a functional human TIMP-2 in tumor cells transduced with the TIMP-2-containing vector was documented. Furthermore, histological analysis of tumors derived from tumor cells co-injected with the TIMP-2 vector producer cells revealed the presence of a thick connective tissue capsule and a lack of local invasion. The data indicate that retroviral-mediated transduction of TIMP-2 cDNA into a limited population of tumor cells in vivo is sufficient to increase the accumulation of connective tissue proteins in tumor tissue, to inhibit growth, and to prevent local invasion.

Introduction

MMPs consist of a family of Zn+-dependent neutral endoproteases that have a broad spectrum of proteolytic activity for most components of the ECM (1). In the extracellular space, the activity of these proteases is regulated by a specific class of natural inhibitors, designated TIMPs (2). The balance between MMPs and TIMPs is critical in maintaining the integrity of the ECM, and its regulatory role in organ development, cell growth, and differentiation has been well documented (3). During tumor invasion, the balance between MMPs and TIMPs is often shifted in favor of the proteases (4), resulting in an excessive proteolytic degradation of the ECM. It has been demonstrated that alteration of this balance in tumor cells by genetic manipulation has a significant effect on tumor progression and tumor growth (5–7). We have demonstrated that overexpression of TIMP-2 in tumorigenic and invasive c-Ha-ras-transfected rat embryo fibroblasts (4R) injected s.c. in nude mice not only limits experimental metastasis but also inhibits the growth of the primary tumors in vivo and results in the formation of a peritumoral capsule of connective tissue that prevents local invasion (8). A similar growth-inhibitory effect of TIMP-2 overexpression was observed in human melanoma cells injected s.c. in SCID mice, and this inhibitory effect was found to be dependent on the integrity of the collagen matrix (9). Altogether, these observations indicate that genetic manipulation of the protease-protease inhibitor balance in tumors in favor of the inhibitors may have a significant cytostatic effect in cancer.

Over the last 5 years, it has been suggested that genetic manipulation in tumor cells by gene therapy may provide an alternative to conventional approaches for the treatment of cancer. Cytotoxic genes such as the herpes simplex-thymidine kinase suicide gene (10), tumor suppressor genes (11), and immunostimulatory cytokine genes (12) have been among the first candidates to be tested in animal models, and some of them are currently being tested in a variety of human gene therapy trials for cancer (13). A major limiting factor in cytotoxic suicide and tumor suppressor genes is that they have to be targeted to virtually every single tumor cell by a combination of direct gene transfer and bystander effect to be able to stop growth of the malignant cells (10). Thus, in this aspect, protease inhibitor genes may represent attractive candidates in a gene therapy approach to cancer because it may not be necessary to deliver and express these genes in every single tumor cell as long as the level of expression in a limited number of transduced cells is sufficient to prevent the excessive breakdown of the ECM. To test this hypothesis, we have overexpressed TIMP-2 in tumor cells in vivo by retroviral-mediated gene transfer, and we demonstrate a significant inhibitory effect on growth and local invasion.

Materials and Methods

Construction of Retroviral Vectors. A Nco-Stu fragment of the human TIMP-2 cDNA, extending from the ATG codon to 5 bp downstream of the stop codon (8) was inserted into plasmid PIC20H to generate PIC20H-TIMP-2. From this plasmid, a 735-bp HindIII fragment was further subcloned into retroviral vector plasmid, LNCX, to generate LNCTIMP-2. In this plasmid, the 5’ Moloney murine leukemia virus LTR is driving expression of the bacterial neomycin resistance (neo) gene, and the CMV promoter is driving expression of the TIMP-2 cDNA (Fig. 1A).

Generation of Retroviral Vector-producing Packaging Cells. The retroviral vector LNCTIMP-2 was transfected into the murine GP+E-B6 ecotropic packaging cell line (14) by lipofection using N-[1-(2,3)-dioleoyl-oxylpropyl]-N,N,N-trimethylammoniummethylsulfate transfection reagent (DOTAP, Boehringer-Mannheim, Indianapolis, IN). The transiently produced retroviral particles were collected from the culture medium of these cells after 48 h and were used to infect the murine PA317 amphotropic packaging cell line (15). Transduced cells were then selected in the presence of 0.5 mg/ml of
active G418 (Geneticin; Life Technologies, Inc., Grand Island, NY), and individual G418-resistant clones were isolated and expanded. The viral titer of the PA317 clone was measured by transfer of G418 resistance to NIH3T3 cells incubated in the presence of serial dilutions of viral supernatant. A vector containing the Escherichia coli ß-galactosidase (LacZ) gene (LlacZSN) provided as a high titer (10^8 colony-forming units/ml) PA317 packaging cell line by Dr. A. D. Miller (The Fred Hutchinson Cancer Research Center, Seattle, WA) was used as a control.

**In Vivo Tumorigenic Model.** 4R (8) tumor cells (5 × 10^4) and irradiated (45Gy) retroviral vector producer cells (2.5 × 10^5 and 5 × 10^5) were co-suspended in 0.1 ml of sterile PBS (137 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, and 1.7 mM KH_2PO_4, pH 7.4) and injected s.c. into each flank of 6-8-week-old female athymic (nu/nu) mice at a tumor cell:producer cell ratio of 1:5 and 1:10. The volumes of these s.c. growing tumors were then calculated over time from three dimensional measurements using a caliper. After 18 days, the animals were sacrificed, and the tumors and the underlying muscle layer were resected. Several 1-mm^3 fragments of each tumor were placed in a tissue culture plate for growth ex vivo; one 2-3-mm^3 fragment was snap-frozen in liquid nitrogen for subsequent RNA extraction, and the remaining tissue was fixed in 10% (v/v) buffered formalin and embedded in paraffin for histological analysis. Sagittal sections (5 μm) of each tumor were stained with H&E and Masson Trichrome and analyzed for the presence of muscular invasion and connective tissue deposition. Expression of TIMP-2 in these sections was examined by in situ hybridization using [33P]UTP-labeled single-stranded TIMP-2 RNA prepared using a Boehringer SP6T7 transcription kit. Hybridization was performed according to the modified method of McLaughlin and Margolskee (16). 

**In Vivo Transduction with LlacZSN Retroviral Vector.** Sections were washed in cold PBS and fixed in 0.5% glutaraldehyde at room temperature for 5 min. Slides were then washed twice with PBS at 4°C and incubated for 4 h in X-Gal solution (1.3 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, and 1.7 mM KH_2PO_4, pH 7.4) and incubated at 4°C for 24 h. The slides were washed in cold PBS and fixed in 0.5% glutaraldehyde at room temperature for 5 min. Slides were then washed twice with PBS at 4°C and incubated for 4 h in X-Gal solution (1.3 mM MgCl_2, 15 mM NaCl, 44 mM Hepes buffer, pH 7.4, containing 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, and 2% X-Gal in N,N-dimethylformamide). Cells harvested ex vivo from these tumor explants were stained similarly with X-Gal.

**Northern Blot Analysis.** Total cellular RNA samples (10 μg) were electrophoresed in 1% (v/v) agarose gels containing formaldehyde and transferred to nylon membranes (ICM Biocuments, Aurora, OH). Blots were sequentially hybridized at 42°C in the presence of [32P]-labeled cDNA probes for TIMP-2, neomycin resistance (neo^R), and GAPDH.

**Reverse Zymogram.** Reverse zymograms were used to detect the presence of functional metalloproteinase inhibitors in the culture medium of tumor cells harvested ex vivo (17). Briefly, gelatin (0.1%) and p-aminoephillicarbaldehyde-activated crude collagenase derived from rabbit fibroblasts were incorporated into the polyacrylamide mixture prior to polymerization. After electrophoresis at 4°C, the gels were washed in Triton X-100 (2.5%) to remove SDS and incubated overnight at 37°C in 50 mM Tris-HCl, pH 7.5, containing 200 mM NaCl and 10 mM CaCl_2. The gels were then stained with 0.25% Coomassie Brilliant Blue and destained in methanol:acetic acid:water (50:10:40). A dark zone indicates the presence of an inhibitor of gelatinases. A semiquantitative comparison of the amount of TIMP-2 present was done by gel scanning, using a Beckman Appraise densitometer system.

**Results**

**Generation and Characterization of a PA317 Clone Producing the LNCTIMP-2 Retroviral Vector.** Twenty G418-resistant clones of PA317 amphotropic packaging cells transfected with the LNCTIMP-2 retroviral vector plasmid were established. Among those, three clones, designated 7, 8, and 21, with viral titers of 2 × 10^5, 5 × 10^5, and 3 × 10^5 colony-forming units/ml, respectively, were selected and analyzed for the expression of vector-derived TIMP-2. PA317/LNCTIMP-2 clone 8 was further selected for in vivo experiments because it had the highest titer and expressed high levels of a 1.5-kb TIMP-2 mRNA driven by the CMV promoter and a 4.5-kb mRNA driven by the LTR promoter, which overlapped with the 3.8-kb, endogenous TIMP-2 mRNA (Fig. 1B). In addition, this clone was found to transduce HeLa cells in vitro, which expressed the 1.5-kb CMV promoter driven TIMP-2 mRNA, and secreted a 2.2-fold increased amount of a functional TIMP-2 (Fig. 1C).

**Effect of TIMP-2 on Tumor Growth.** PA317/LNCTIMP-2 cells were used to induce TIMP-2 overexpression in tumor cells in vivo in a mouse xenograft model. In these experiments, 4R cells were co-injected s.c. in nude mice in the presence of irradiated LNCTIMP-2 vector producer cells using tumor cell:producer cell ratios of 1:5 and 1:10. As a control, 4R cells were co-injected with LlacZSN vector producer cells, which also allowed estimation of in vivo transduction...
The use of protease inhibitors as cytostatic agents for the treatment of cancer has recently been the subject of increased interest. Although it was initially suggested that protease inhibitors could be important agents to suppress or prevent disseminated spread of cancer, recent observations have indicated that these inhibitors can have a significant

efficiency. The growth of these tumors was then monitored over 18 days. The data (Fig. 2) indicate that tumors derived from 4R cells co-injected with LNCTIMP-2 vector producer cells at both ratios grew at a significantly lower rate and, at day 18, had formed smaller tumors (average tumor size, 1.5 cm³) in comparison with the control (average tumor size, 4.1 cm³).

Expression of TIMP-2 in Transduced Tumor Tissue. The expression of TIMP-2 in samples derived from tumors co-injected with retroviral vector producer cells was examined by Northern blot analysis. The data indicate the presence of a 1.5-kb CMV promoter-driven human TIMP-2 mRNA in all tumors examined, in addition to the presence of a 4.5-kb LTR promoter-driven mRNA. In contrast, tumors in the control group only expressed the two endogenous TIMP-2 mRNAs of 3.8 and 1.2 kb (Ref. 18; Fig. 3A). Reverse zymogram analysis was performed on the conditioned medium obtained from several cultures derived from these tumors and maintained without G418 for 4 days. This analysis demonstrated an increase in the amount of functional TIMP-2 secreted, ranging from 1.2- to 1.8-fold in all G418 unselected cultures tested (Fig. 3B).

Transduction Efficiency. The transduction efficiency was first examined in tissue sections of tumors derived from 4R cells co-injected with LLacZSN vector producer cells (tumor cell:producer cell ratio of 1:5) stained with X-Gal. This analysis revealed the presence of blue cells evenly distributed within the tumor mass (Fig. 4A). A quantitative analysis performed in vitro in cultures derived from these tumors revealed a transduction efficiency of 13% (±1.8; Fig. 4B).

Evidence for in Vivo Gene Transfer into Tumor Cells. To eliminate the possibility that the expression of TIMP-2 in these tumors was derived from the irradiated PA317 packaging cells rather than the transduced tumor cells, we also examined tumor tissues for the presence of the amphotropic retroviral envelope gene present in the packaging cells by reverse transcription-PCR using oligonucleotides coding for the amphotropic env gene. The data (not shown) indicate an absence of detection of the env gene in all of the tumor specimens examined, suggesting, therefore, that these cells did not survive after 18 days in vivo.

Effect of TIMP-2 Expression on Local Invasion. The effect of enhanced TIMP-2 expression on tumor invasion was examined on histological sections of tumors stained with H&E and Masson Trichrome. In all tumors derived from 4R cells co-injected with LNCTIMP-2 vector producer cells, the tumor cells were found confined to the s.c. layer and failed to invade the underlying muscle (Fig. 4D). Multiple sections obtained in these tumors consistently revealed the presence of a thick connective tissue capsule rich in collagen that prevented invasion into the muscle, as indicated by the intense blue staining obtained with the Masson Trichrome (Fig. 4F). In contrast, tumors derived from 4R cells co-injected with LLacZSN vector producer cells had invaded the muscle of the abdominal wall and often had penetrated the peritoneal cavity (Fig. 4C). In these tumors, there was very little evidence for collagen deposition, as indicated by the discrete blue staining obtained with Masson Trichrome (Fig. 4E). Expression of TIMP-2 in these tumors was monitored by in situ hybridization in the presence of a [35S]UTP-labeled antisense riboprobe for human TIMP-2 and compared with tumors derived from 4R cells transfected in vitro and selected for TIMP-2 overexpression. Whereas analysis of tumors transfected in vitro showed the presence of a strong signal through the entire tumor tissue (Fig. 4G), analysis of tumors transduced in vivo revealed the presence of isolated positive cells scattered through the entire tumor tissue (Fig. 4H). No signal was detected when a sense riboprobe was used (Fig. 4I).

Discussion

The use of protease inhibitors as cytostatic agents for the treatment of cancer has recently been the subject of increased interest. Although it was initially suggested that protease inhibitors could be important agents to suppress or prevent disseminated spread of cancer, recent observations have indicated that these inhibitors can have a significant
effect on tumor growth in vivo (8, 19, 20). For example, a synthetic inhibitor of metalloproteinases, BB94, has been shown to inhibit local invasion and growth of ovarian and colon carcinoma cells when injected intraabdominally in immunosuppressed mice (21, 22). Although synthetic micromolecular inhibitors offer the advantage that they can be synthetically modified to achieve higher specificity and affinity, their requirement for systemic administration implies good solubility properties and is associated with the risk of systemic toxicity. In contrast, natural inhibitors such as TGFs offer the advantage that their expression in tumors could be genetically manipulated, and their effect could therefore, remain local.

We had demonstrated previously that overexpression of an inhibitor of metalloproteinases, TIMP-2, in tumor cells has a cytostatic effect on tumor growth and prevent local invasion (8). We had also demonstrated that a growth-inhibitory effect of TIMP-2 could be achieved in vitro by growing cells in a three-dimensional collagen gel (9). Therefore, we postulated that the inhibitory effect of TIMP-2 was a result of its ability to prevent the degradation of the ECM. Because in these experiments, cells overexpressing TIMP-2 were selected in vitro, it had remained unknown whether a similar inhibitory effect could be achieved if only a small percentage of the population of tumor cells were overexpressing the inhibitor. Using the β-galactosidase gene as a marker transduced gene, we found that approximately 13% of the tumor cells was effectively transduced. Since the titer of the LacZ vector producer cells was 2-fold higher than the titer of LNCTIMP-2 vector producer cells, it is likely that the transduction efficiency of this latter vector was even lower. Whether delayed injections into established tumors will result in a similar transduction efficiency, however, remains to be determined. Our data clearly show that despite the transduction of a relatively small number of tumor cells, the amount of inhibitor secreted into the tumor tissues is sufficient to inhibit growth and limit local invasion. This is consistent with our hypothesis that TIMP-2 exerts its inhibitory effect through its protective action on the ECM, as clearly demonstrated by the presence of an increased amount of collagen deposition in the tumors transplanted with TIMP-2. Because it will be possible to design vectors that could be specifically targeted to tumors, this approach may have potential value to inhibit the growth of primary tumors and established metastatic lesions.

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References

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