

# Down-Regulation of Vascular Endothelial Growth Factor by Tissue Inhibitor of Metalloproteinase-2: Effect on *in Vivo* Mammary Tumor Growth and Angiogenesis<sup>1</sup>

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## Abstract

The tissue inhibitor of metalloproteinases-2 (TIMP-2) has at least two independent functions, *i.e.*, regulation of matrix metalloproteinases and growth promoting activity. We investigated the effects of TIMP-2 overexpression, induced by retroviral mediated gene transfer, on the *in vivo* development of mammary tumors in syngeneic mice inoculated with EF43.*fgf-4* cells. The EF43.*fgf-4* cells established by stably infecting the normal mouse mammary EF43 cells with a retroviral expression vector for the *fgf-4* oncogene, are highly tumorigenic and overproduce vascular endothelial growth factor (VEGF). Despite a promotion of the *in vitro* growth rate of EF43.*fgf-4* cells overexpressing *timp-2*, the *in vivo* tumor growth was delayed. At day 17 post-cell injection, the volume of tumor derived from TIMP-2-overexpressing cells was reduced by 80% as compared with that obtained with control cells. Overexpression of TIMP-2 was associated with a down-regulation of VEGF expression *in vitro* and *in vivo*, a reduction of vessel size, density, and blood supply in the induced tumors. In addition, TIMP-2 completely inhibited the angiogenic activity of EF43.*fgf-4* cell-conditioned medium *in vitro* using a rat aortic ring model. Our findings suggest that overexpression of TIMP-2 delays growth and angiogenesis of mammary carcinoma *in vivo* and that down-regulation of VEGF expression may play an important role in this TIMP-2-mediated antitumoral and antiangiogenic effects. Finally the *in vivo* delivery of TIMP-2, as assessed by *i.v.* injection of recombinant adenoviruses vectors, significantly reduced the growth of the EF43.*fgf-4*-induced tumors. This effect of TIMP-2 was shown to be equally comparable with that of angiostatin, a known potent inhibitor of angiogenesis.

**Abbreviations** : ECM, extracellular matrix; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; VEGF, vascular endothelial growth factor; rTIMP, recombinant TIMP; RT-PCR, reverse transcription-PCR; pfu, plaque-forming unit(s)

## INTRODUCTION

Angiogenesis is a pivotal step during cancer progression because it promotes both primary tumor growth and metastasis dissemination (1). It is a multistep process characterized by (a) the activation of vascular endothelial cells by angiogenic factors; (b) migration of endothelial cells toward the source of chemotactic stimuli; (c) degradation of the ECM<sup>32</sup> to allow endothelial cell invasion into surrounding tissue; and (d) differentiation of endothelial cells into microvessels and stabilization (2, 3). The matrix breakdown is mediated by ECM-degrading enzymes including MMPs secreted, at least by endothelial cells, in response to angiogenic stimuli. MMPs form a large family of structurally related zinc endopeptidases (4, 5) that are collectively able to degrade

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a wide variety of ECM proteins. MMP activity is tightly regulated by proenzyme activation and by the action of specific physiological inhibitors, the tissue inhibitors of metalloproteinases or TIMPs. The TIMP family comprises at least four distinct members, TIMP-1, TIMP-2, TIMP-3, and TIMP-4 (reviewed in Refs. 6,7). TIMP-2 is a  $M_r$  21,000 nonglycosylated protein that forms a 1:1 complex with the latent and activated form of MMP-2 (8). It is also known to interact with the activated form of different MMPs including MMP-9 (9).

Recent studies have shown that MMP-deficient mice exhibit delayed or reduced angiogenic responses during embryonic development or in response to tumor xenograft (10, 11). More direct evidence of the MMP involvement in the angiogenic process is that MMP inhibitors, both synthetic and physiological, inhibit angiogenesis *in vitro* and *in vivo* (2, 12). TIMP-2 blocks neoangiogenesis in collagen or fibrin matrix (13, 14). Furthermore, a number of reports have demonstrated a TIMP-2-mediated inhibition of tumor growth and invasion in murine models, when administrated exogenously, as well as overexpressed through *timp-2* cDNA transfection of different tumor cell lines (15-18). However, high tissue concentration in TIMP-2 are associated with poor prognosis in breast carcinomas (19). This paradoxical clinical observation can be partly explained by the multiple functions of TIMP-2. Indeed, TIMP-2 not only exhibits a broad specificity of inhibition against MMPs but also modulates cell properties such as proliferation and apoptosis (7, 20). Also, although TIMP-2 inhibits MMP-2 activity at high concentrations, it also promotes its activation at low concentrations and in association with MT-MMPs (21, 22).

We have previously demonstrated that the expression of the *fgf-4* oncogene in normal murine mammary EF43 cells yielded a cell population that induced *in vivo* rapidly developing and highly vascularized tumors (23, 24). The angiogenic potential of these cells was attributed to their capacity to produce VEGF, as demonstrated *in vitro* (25). In the present study, we induced the overexpression of TIMP-2 by introducing *timp-2* cDNA into these murine EF43 *fgf-4* cells by retroviral mediated gene transfer. This led to a delay of *in vivo* tumor growth and angiogenesis. This antiangiogenic effect of TIMP-2 observed both *in vitro* and *in vivo* was associated with a down-regulation of VEGF expression by tumor cells. All together, these results emphasize the potency of TIMP-2 as an antiangiogenic agent and provide a new possible mechanism of TIMP-2 action, through the down-regulation of VEGF expression.

## MATERIALS AND METHODS

### Cell Cultures and TIMP-2 Analysis

The normal mouse mammary EF43 cell line and its tumorigenic derivatives, the EF43 *fgf-4* cells (23), were grown at 37°C in 5% CO<sub>2</sub> in DMEM supplemented with 10% fetal bovine serum (Life Technologies, Inc.), penicillin (100 units/ml), and streptomycin (100 µg/ml).

To measure their proliferation rate *in vitro*, cells ( $2 \times 10^4$ ) were seeded in quadruplicate in 24-well plates in complete medium for 48 h and were then cultured in serum-depleted medium for 7 days. DNA content was evaluated daily by fluorimetry (26).

To prepare conditioned medium, cells were cultured to confluence, washed twice with serum-free medium and covered, with 2 or 5 ml of fresh serum-free medium in 3.3- or 10-cm diameter dishes, respectively. Conditioned medium was collected 24 h later and clarified by centrifugation or filtration through a 0.45 µm filter. The results were corrected for cellular DNA content measured by fluorimetry in each culture dish. TIMP-2 amounts in the conditioned media were measured by ELISA as described previously (15, 19, 22).

When indicated, human rTIMP-2, purified as described previously (15, 27), was added to the culture medium every 2 days.

### Retroviral Vector Construction and Production

The *timp-2*-carrying retroviral plasmid was constructed as described previously (15). It contains the human *timp-2* sequence cDNA under the control of viral 5'-LTR sequence, and a selection gene (puro conferring resistance to puromycin) driven by an internal SV40 early promoter. Production of retroviruses with titers of 1 to  $5 \times 10^5$  cfu/ml and cell transduction were performed as described previously (23). The EF43 *fgf-4.C* and EF43 *fgf-4.timp-2* cells were populations of cells resistant to puromycin (1 µg/ml) and containing the control empty vector or the *timp-2* cDNA-carrying vector, respectively.

## Analyses of VEGF Expression

### *Northern Blotting.*

Total cellular RNA was purified as described previously (28), with slight modifications as described elsewhere (22). RNA samples (15  $\mu$ g) were run on 1% agarose gels containing 10% formaldehyde and transferred onto nylon membranes (Hybond-N; Amersham, Aylesbury, United Kingdom). The probe used was the murine VEGF<sub>164</sub> cDNA generously provided by P. D'Amore (Laboratory for Surgical Research, Children's Hospital, Boston, MA). Hybridization was performed according to the procedure described previously (25).

### *RT-PCR.*

Total RNA was extracted from cell layers or tumors by RNA Instapure treatment (Eurogentec, Liège, Belgium). RT-PCR was performed on 10 ng of total RNA using a Perkin-Elmer kit (Foster City, CA) and following the manufacturer's instructions. RT was carried out with 5'-CTC ACC GCC TCG GCT TGT CAC A-3' as primer during 15 min at 70°C. PCR products were generated with the same primer as reverse primer and with 5'-CCT GGT GGA CAT CTT CCA GGA GTA-3' as forward primer. PCR conditions were 95°C for 2 min, followed by 29 cycles consisting of 94°C for 20 s, 66°C for 20 s, 72°C for 30 s, and a final elongation step of 72°C for 2 min. The amplification products of the RNAs coding for VEGF<sub>188</sub>, VEGF<sub>164</sub>, and VEGF<sub>20</sub>, were 479, 407, and 265 bp sizes, respectively. To control the efficiency of the RT-PCR, we designed a synthetic RNA (CTR1) that could be reverse transcribed and amplified with the same primers and giving rise to a 311-bp fragment. A total of 8000 copies of this internal control were added to each sample. VEGF expression was normalized to that of the 28S. PCR conditions for 28S were 95°C for 2 min, 17 cycles consisting of 94°C for 15 s, 68°C for 20 s, 72°C for 10 s, and a final elongation step of 72°C for 2 min. A synthetic RNA (CTR2) was also used to control the efficiency of RT-PCR for 28S. The amplification products were electrophoresed on a polyacrylamide gel, stained with Gelstar (Sanver Tech, Antwerpen, Belgium), scanned with FluorSImager, and analyzed using multianalyst software (Bio-Rad, Belgium).

Ratios VEGF:CTR1 and 28S:CTR2 were determined. VEGF expression was expressed as the ratio of VEGF transcripts/28S transcripts.

### *Western Blot Analysis in Tumors.*

Tumors were frozen in liquid nitrogen, powdered, extracted with lysis buffer (0.1 M Tris-HCl/0.4% Triton X-100), and centrifuged at 12,000 rpm. Samples containing 15  $\mu$ g of protein were electrophoresed on a 15% SDS-polyacrylamide gel. Western blotting was performed as described previously (25), using a 1:500 dilution of a monoclonal antibody against human VEGF165 and mouse VEGF164 (V-4758; Sigma) and, as secondary antibody, peroxidase-conjugated goat antimouse IgG (Dako; P0447) diluted 1:1000. Peroxidase was revealed by the enhanced chemiluminescence assay (Amersham Corp).

## Tumorigenicity and Antitumoral Effect of Adenoviral Vectors

The cells were trypsinized, counted, centrifuged, and resuspended in serum-free medium. EF43 *fgf-4.timp-2* and EF43 *fgf-4.C* control cells ( $5 \times 10^4$ ,  $10^5$ ,  $5 \times 10^5$ , and  $10^6$ ) were injected s.c. into syngeneic 4-week-old female BALB/c mice. Tumor growth was monitored twice a week by caliper measurement of 2 diameters, and expressed as mean tumoral volume  $\pm$  SD. In each assay, the number of mice per group (*n*) was 10.

To evaluate the antitumoral effect of a TIMP-2 treatment on the EF43 *fgf-4* tumor growth, we used adenoviral vectors (Ad.*timp-2*) that carry a cytomeg-alo virus-driven cDNA for human TIMP-2. To obtain this adenovirus, the TIMP-2 cDNA was inserted in pC05 shuttle vector at the downstream of cytomegalovirus early gene promoter (pCMV). The SV40 late gene poly-adenylation signal sequence was inserted after the stop codon of cDNA. The recombinant adenovirus was obtained in 293 cells by homologous recombination between shuttle vector containing TIMP-2 expression cassette and E1/E3-deficient human type 5 AdRSV. $\beta$ gal DNA (29). The viruses ( $5 \times 10^9$  pfu) were injected into the tail vein of mice (*n* = 7) bearing a 20-mm<sup>3</sup> preestablished tumor. Control mice with the same tumor size (*n* = 7) were similarly injected with AdCO1 vectors that carried the empty expression cassette only. Vectors carrying the  $\beta$ -galactosidase gene, Ad. $\beta$ -gal (30) were also injected into mice to confirm the adenoviral vector expression. At the end of the experiment, the livers from all of the animals were positive for  $\beta$ -galactosidase staining. Adenoviral vectors, AdK3 (30) carrying a cDNA for the NH<sub>2</sub>-terminal fragment from human plasminogen, including the first three kringle domains (angiostatin K3), ( $5 \times 10^9$  pfu) were also injected i.v. under the same conditions.

## Evaluation of Tumor Vascularization

The extent of tumoral vascularization was assessed by immunostaining using a rabbit polyclonal serum raised against laminin (diluted 1:100; Ref 31) or a rat monoclonal antibody raised against PECAM (PharMinogen, San Diego, CA; diluted 1:20). Frozen sections (6- $\mu$ m) of tumor tissues were fixed in paraformaldehyde 4% for 5 min and then were incubated with the primary antibodies for 60 min at room temperature. After three washes with PBS, the sections were incubated with fluorescein-conjugated swine antirabbit (Dako; dilution 1:50) for 30 min. Preparations were mounted with Eukitt (Labonord, Villeneuve, France) and viewed with an Olympus microscope.

Vessel density was evaluated under light microscopy on sections of whole tumors. Ten sections were made in each tumor. Image analysis and quantification of stained vessel sections were performed on a computer using the Microimage 3.0.1.0. software from Olympus (Bio-Rad, Belgium). Results were expressed as mean ( $\pm$  SD) vessel number per  $\text{mm}^2$ .

## *In Vitro* Angiogenesis: the Aorta Ring Assay

Rat aortic explant cultures were prepared as described previously (32). For the preparation of culture wells, 30 ml of 1.5% agarose solution (type VII, cell culture-tested; Sigma, St. Quentin Fallavier, France) were poured into 100-mm-diameter Petri dishes (Corning Costar Corporation, Cambridge, MA) and allowed to gel. Agarose rings were obtained by punching two concentric circles in the agarose with punchers of 10- and 17-mm diameter and were transferred to additional 100-mm diameter Petri dishes (bacteriological polystyrene; Falcon; Becton Dickinson, Lincoln Park, NJ). The bottom of each agarose well was coated with 200  $\mu$ l of rat tail tendon (type I) collagen (1.5 mg/ml; Collagen R; Serva, Heidelberg, Germany) that was allowed to gel at 37°C. One aortic ring obtained by sectioning the rat aorta at 1-mm intervals (32), was carefully positioned in each well, which was then completely filled with collagen solution. Aorta rings were maintained at 37°C in MCDB131 (Life Technologies LTD., Paisley, Scotland) supplemented with 25 mM  $\text{NaHCO}_3$ , 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cultures were examined every 2 days by phase-contrast microscopy. When indicated, human recombinant VEGF<sub>165</sub> (20 ng/ml; Sigma) was added to the medium. The identity of endothelial cells was verified by Dil-Ac-LDL incorporation in the aorta ring before embedding into collagen gel (33).

## RESULTS

### *In Vitro* Development of Cells Overproducing TIMP-2.

The highly tumorigenic EF43.*fgf-4* cells were infected with retroviral vectors carrying the *timp-2* cDNA or the control vectors carrying only the *puro* selection gene. Stable transformants derived from *timp-2* cDNA transduction (EF43.*fgf-4.timp-2* cells), and cell populations derived from control retroviral vector transduction (EF43.*fgf-4.C* cells) were obtained by puromycin selection.

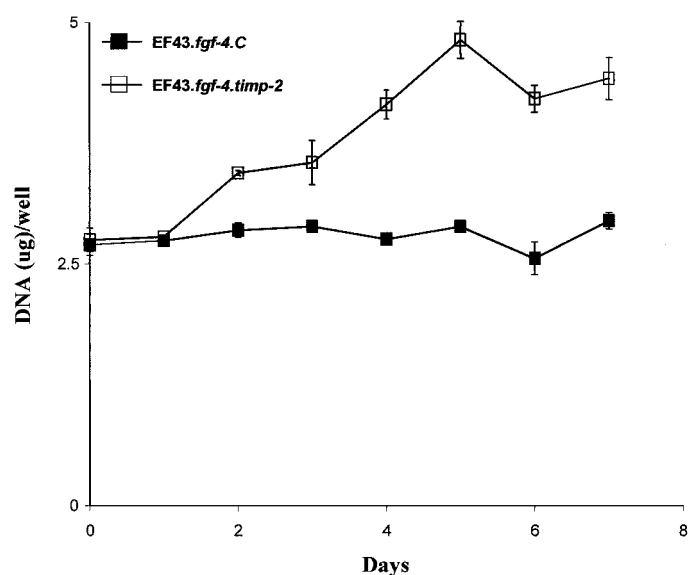
The serum-free media conditioned by these different cell populations were analyzed for the presence of TIMP-2 by ELISA. Endogenous TIMP-2 production was weak in control EF43.*fgf-4.C* cells ( $0.13 \pm 0.02$  ng/ $\mu$ g DNA), whereas it was increased in EF43.*fgf-4.timp-2* cells ( $0.98 \pm 0.09$  ng/ $\mu$ g DNA). The FGF-4 production was not modulated by the increased TIMP-2 secretion as determined by Western blot (data not shown).

Because it was reported that TIMP-2 can stimulate the growth of some cell types (7, 34), we compared the *in vitro* growth rate of EF43.*fgf-4.timp-2* cells and EF43.*fgf-4.C* cells in serum-free medium. EF43.*fgf-4.C* cells did not multiply in these serum-free conditions, whereas overexpression of *timp-2* significantly promoted the proliferation of EF43.*fgf-4* cells (Fig. 1).

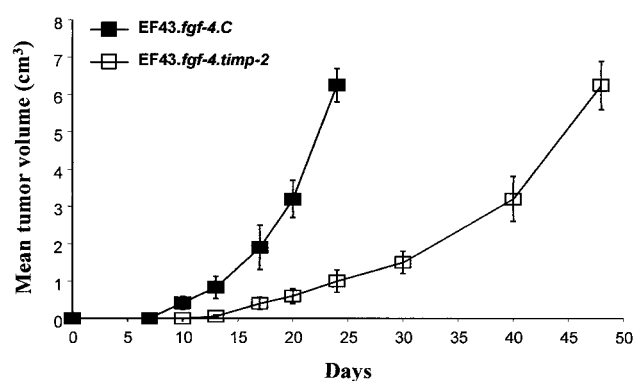
### *In Vivo* Development of Tumoral Cells Overproducing TIMP-2.

We previously reported that the EF43.*fgf-4* cells were highly tumorigenic when injected s.c. into syngeneic BALB/c mice, leading to tumor onset within 1 week (23, 24). Here, we show that injection of both EF43.*fgf-4.C* and EF43.*fgf-4.timp-2* cells led to tumor formation, but the growth of tumors induced by EF43.*fgf-4.timp-2* cells was strikingly delayed (Fig. 2). At day 17 postimplantation, the volume of these tumors was reduced by 80% as compared with the ones induced in EF43.*fgf-4.C* control tumors. Fig. 2 illustrates an experiment based on the injection of  $5 \times 10^5$  cells. Similar delays in tumor development were observed after inoculation of  $5 \times 10^4$ ,  $10^5$ , or  $10^6$  cells (data not shown).

**Fig. 1:** *In vitro* cell growth rate. The EF43.fgf-4.C and EF43.fgf-4.timp-2 cells ( $2 \times 10^4$ ) were cultured in serum-free medium as described in "Materials and Methods". Cell proliferation was expressed as mean  $\pm$  SD DNA amount/well of quadruplicate cultures



**Fig. 2:** *Tumorigenicity.* Tumors were induced by s.c. injection of  $5 \times 10^5$  EF43.fgf-4.C or EF43.fgf-4.timp-2 cells in BALB/c syngeneic mice. Symbols, mean tumor volumes ( $\text{cm}^3$ )  $\pm$  SD.



When observed macroscopically directly after resection, the EF43.fgf-4.C tumors appeared more vascularized than their EF43.fgf-4.timp-2 counterparts. Large-size blood vessels were closely associated with the edge of tumor derived from EF43.fgf-4.C cells. In contrast, EF43.fgf-4.timp-2 tumors displayed only a weakly developed peripheral neovascularization. In addition, during tumor growth, hemorrhagic areas appeared regularly inside the EF43.fgf-4.C tumors. Such vascular ectasias and blood extravasation were always absent from the EF43.fgf-4.timp-2 tumors, at any stage of development (Fig. 3).

Tumors grown from EF43.fgf-4.C cells appeared as dense adenocarcinoma (Fig. 3, A1 and A2) with a clearer zone formed by large vascular cavities. At higher magnification, a well-organized network of anastomosing vessels was seen in these tumors (Fig. 3, A3). At a more advanced stage, we observed an angiomatoid pattern characterized by cellular necrosis and the presence of large vessels with a dilated lumen, as well as large vascular cystic cavities filled with blood (Fig. 3, A4). The large size vessels were always limited by a subendothelial basement membrane containing laminin (Fig. 4C). On the other hand, tumors derived from EF43.fgf-4.timp-2 cells essentially appeared at all stages as dense adenocarcinoma (Fig. 3, B1 and B2), but well-defined vessels were observed with a narrow lumen or even collapsed, probably caused by the pressure of the dense tumoral mass (Fig. 3, B3 and B4). These tumors were devoid of vascular cavities, and the amount of blood was greatly

reduced as compared with control *EF43.fgf-4.C* tumors.

#### *Vascular Density in Tumors Overproducing TIMP-2.*

Tumor vascularization was thus measured on tumor sections immunostained with an antilaminin antibody (Fig. 4). Tumors were compared when they had reached the same volume of 0.4 cm<sup>3</sup>, 3.2 cm<sup>3</sup>, and 6.25 cm<sup>3</sup>. Such volumes were reached 10, 20, and 24 days after implantation of the *EF43.fgf-4.C*, respectively, and 17, 40, and 48 days after implantation of *EF43.fgf-4.timp-2* cells, respectively (Fig. 2). Tumors formed by *EF43.fgf-4.C* cells harbored numerous long and anastomosing vessels (Fig. 4A). Shorter vessels were observed in tumors derived from *timp-2*-overexpressing cells (Fig. 4B). Such reduction of tumor angiogenesis in *EF43.fgf-4.timp-2* tumors was confirmed by quantitative analysis. Indeed, a significant reduction of vessel density within the *EF43.fgf-4.timp-2* tumors was observed as compared with the *EF43.fgf-4.C* control tumors (Fig. 5). Although the vessel density increased progressively during the growth of *EF43.fgf-4.C* tumors, it remained constant during the evolution of *EF43.fgf-4.timp-2* tumors (Fig. 5).

#### *Effect of TIMP-2 on VEGF Expression.*

We previously reported that *EF43.fgf-4* cells induce *in vitro* angiogenesis through VEGF overexpression (25). We, therefore, evaluated the VEGF expression in cells that overexpressed TIMP-2 or not. Interestingly, Northern blot analysis of total RNA showed a 7.3-fold decrease in VEGF mRNA level in *EF43.fgf-4.timp-2* cells as compared with *EF43.fgf-4.C* cells (Fig. 6, I). By RT-PCR, overexpression of TIMP-2 in *EF43.fgf-4* cells was shown to be associated with more than 5-fold down-regulation of VEGF<sub>188</sub>, VEGF<sub>164</sub>, and VEGF<sub>120</sub> mRNA isoforms (Fig. 6, II). Moreover, the addition of exogenous rTIMP-2 (100 ng/ml) to *EF43.fgf-4.C* cell cultures, resulted in a similar down-regulation of VEGF mRNA isoforms expression (Fig. 6, II). In accordance with these *in vitro* data, a 2- to 3-fold reduction of VEGF mRNA isoforms expression was detected by RT-PCR in *EF43.fgf-4.timp-2* tumors, as compared with tumors obtained from *EF43.fgf-4.C* cells (Fig. 6, III, a). This modulation of VEGF expression was confirmed by measuring the VEGF protein by Western blot analysis (Fig. 6, III, b).

#### *Effect of TIMP-2 on in Vitro Angiogenesis.*

Media conditioned by *EF43.fgf-4.C* or *EF43.fgf-4.timp-2* cells were assayed for their ability to modulate *in vitro* angiogenesis. Rings of rat aorta were embedded in collagen gels and maintained in culture, in the presence of medium conditioned by *EF43.fgf-4.C* or *EF43.fgf-4.timp-2* cells. Microvessels outgrew from rat aorta cultured in the presence of *EF43.fgf-4.C* cell-conditioned medium. In such conditions, the generation of micro vessels was similar to that observed in the presence of 20 ng/ml human recombinant VEGF<sub>165</sub> (Fig. 7). Interestingly, no angiogenic response was observed with medium conditioned by *EF43.fgf-4.timp-2* cells. A similar effect was obtained by adding exogenous rTIMP-2 (100 ng/ml) to the conditioned medium of *EF43.fgf-4* cells.

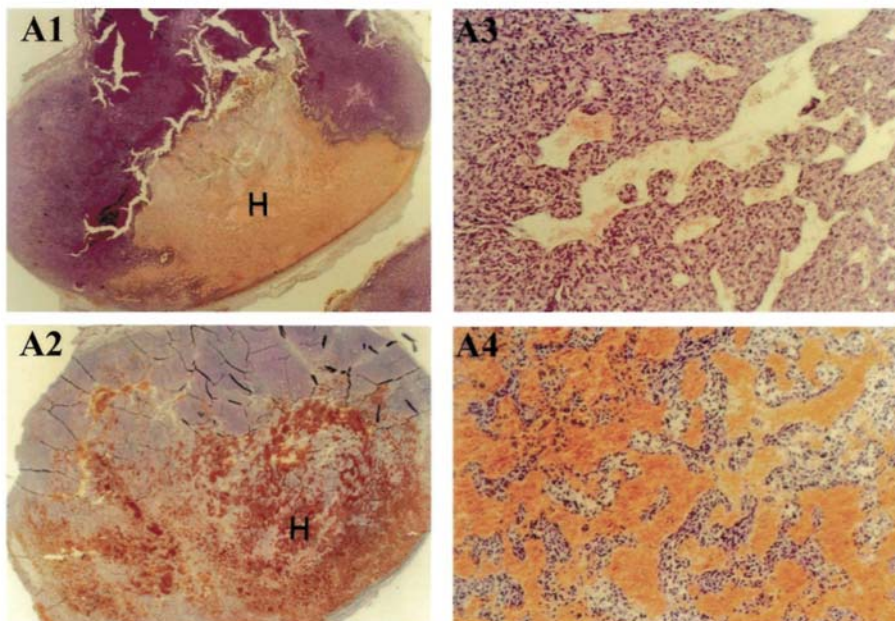
#### *In Vivo Antitumoral Effect of Adenovirus-mediated Gene Transfer of TIMP-2 or Angiostatin.*

To assess potential therapeutic action of *in vivo* TIMP-2 delivery, we used a defective adenovirus (Ad.timp-2) expressing human TIMP-2 under the control of the cytomegalovirus promoter. A single i.v. administration of *Ad.timp-2* ( $5 \times 10^9$  pfu) to mice bearing a 20-mm<sup>3</sup>-preestablished *EF43.fgf-4* tumor resulted in a significant impairment of tumor growth (Fig. 8). Fourteen days after virus injection, the tumoral volume was reduced by 50%. In contrast, a similar injection of control adenoviruses (AdCO1) did not affect tumor growth.

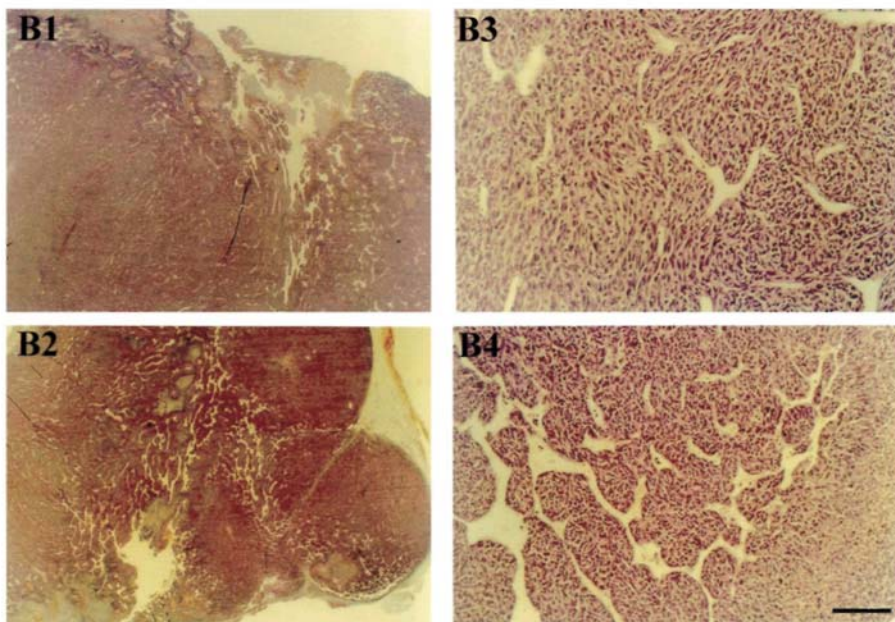
The antitumoral effect of TIMP-2 on *EF43.fgf-4* tumor growth was compared with the effect of a known antiangiogenic factor, angiostatin, identified as the  $M_r$  38,000 NH<sub>2</sub>-terminal fragment of plasminogen. A single i.v. injection of AdK3 ( $5 \times 10^9$  pfu) viruses also exerted an antitumoral effect that was slightly more pronounced than that of TIMP-2, with a 70% reduction in tumor volume at day 14 (Fig. 8).

**Fig. 3:** H&E staining of EF43.fgf-4.C and EF43.fgf-4.timp-2 tumors. Representative tumors obtained after injection of  $5 \times 10^5$  EF43.fgf-4.C cells (A) and EF43.fgf-4.timp-2 cells (B). Sections were performed on tumors with a defined volume of  $3.2 \text{ cm}^3$  and  $6.2 \text{ cm}^3$ , which correspond to 20 (A1, A3) and 24 days (A2, A4) after implantation of the EF43.fgf-4 C cells, and to 40 (B1, B3), and 48 days (B2, B4) after implantation of EF43.fgf-4.timp-2 cells, respectively. Each tumor type is presented at low (A1,A2, B1, B2) and higher (A3, A4, B3, B4) magnifications. Bar,  $800 \mu\text{m}$  (A1, A2, B1, B2) and  $80 \mu\text{m}$  (A3, A4, B3, B4). H, hemorrhagic area

A

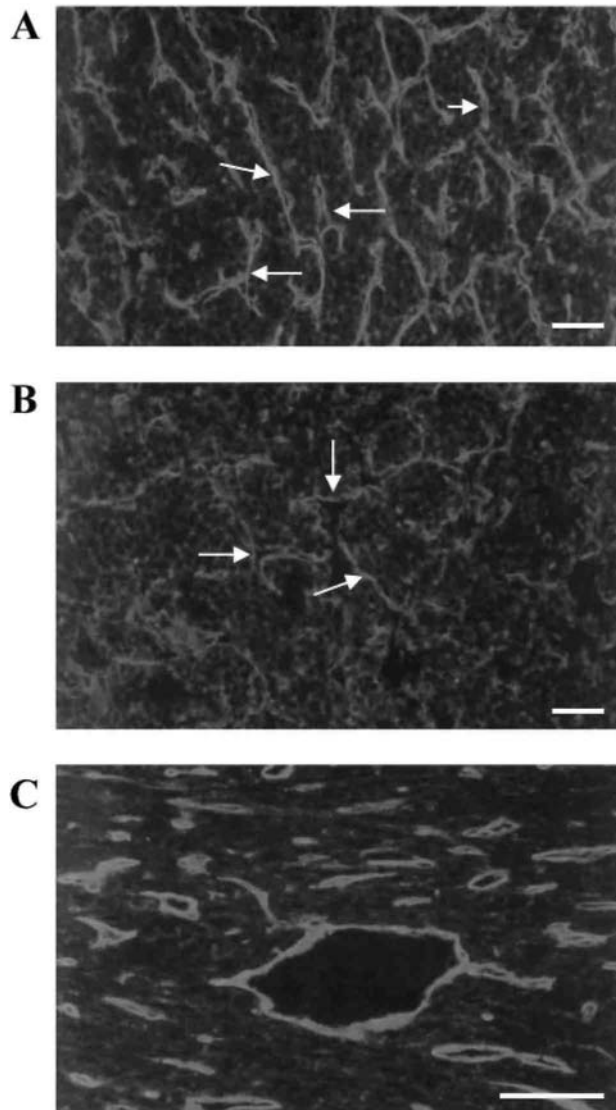


B





**Fig. 4:** Immunostaining of vessels in tumors. Frozen sections of EF43.fgf-4. C (A) or EF43.fgf-4.timp-2 (B) tumors with a volume of 3 cm<sup>3</sup> were stained with antilaminin antibody. C, a section from EF43.fgf-4.C tumor, showing a large vascular cavity limited by a subendothelial basement membrane. Laminin labeling was always codistributed with endothelial cells recognized by the antimouse PECAM antibody (data not shown). White arrows, vessels. Bars, 20 μm



## DISCUSSION

Numerous preclinical studies have demonstrated that synthetic MMP inhibitors inhibit the growth of a variety of tumors in murine xenograft models (reviewed in Refs. 35, 36). On the basis of its inhibitory function against MMPs, it is anticipated that TIMP-2 blocks tumor growth and dissemination. This concept is supported by several reports on experimental models showing a TIMP-2-mediated reduction of tumor growth, invasion, and metastasis (15, 16, 37). Although TIMP-2 overexpression in EF43.fgf-4 cells sustained their proliferation *in vitro*, their *in vivo* growth was considerably reduced and led to 80% tumor growth reduction. TIMP-2 overexpression in tumor cells resulted in: (a) a reduced blood supply as visualized at both microscopic and macroscopic levels; (b) the absence of large dilated vessels and vascular cavities filled with blood on tumor sections; (c) a decrease in vessel density evaluated by immunostaining; and (d) the absence of vascular ectasias. These data demonstrate *in vivo* that TIMP-2 is a potent antiangiogenic agent. The angiogenesis inhibition by TIMP-2 was confirmed *in vitro*, in a rat aorta ring model, by using either medium conditioned by TIMP-2-overexpressing cells or rTIMP-2. This *in vitro* model (32, 38) using intact vascular explants reproduces more



accurately the environment in which angiogenesis takes place than cultures of isolated endothelial cells. The inhibition of angiogenesis by TIMP-2 is consistent with the recent implication of MT1-MMP (14), MMP-9 (10), and MMP-2 (11) in neoangiogenesis.

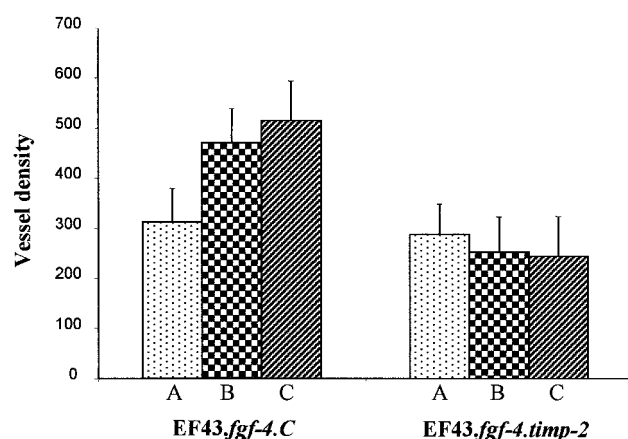
TIMP-2 may inhibit tumor growth and angiogenesis by several mechanisms. We provide evidence for a new mechanism by which TIMP-2 exerts its antitumoral effect: the down-regulation of VEGF expression. This growth factor originally discovered as a permeability factor (VPF; Ref. 39) exhibits both potent endothelial mitogenic and vascular permeability-inducing activity, as well as vessel dilation (3). We previously demonstrated that EF43.fgf-4 cells induced, through the secretion of VEGF, an angiogenic phenotype of endothelial cells in an *in vitro* assay (25). The regulation of VEGF expression by TIMP-2 was here demonstrated *in vitro* and *in vivo*, by Northern blot, RT-PCR, and Western blot analysis. Both endogenous and rTIMP-2 reduced VEGF expression of EF43.fgf-4 cells. The three different mRNA isoforms of VEGF that were expressed at different levels by EF43.fgf-4 cells were all affected by TIMP-2 expression. The mechanisms by which TIMP-2 modulates VEGF expression remain to be elucidated.

All together, these data suggest that TIMP-2 exerts its antiangiogenic effect through different mechanisms including MMP inhibition and down-regulation of VEGF expression. In the aortic ring assay, the antiangiogenic role of TIMP-2 is probably mainly related to its antiproteolytic function independently of its capacity to regulate VEGF expression. Indeed, inhibition of microvessel outgrowth was observed when exogenous rTIMP-2 was added to the EF43.fgf-4 cell supernatant, which contains VEGF. Furthermore, the addition of exogenous rVEGF to the medium conditioned by EF43.fgf-4.timp-2 cells did not bypass the inhibition of angiogenesis (data not shown). As a protease inhibitor, TIMP-2 may control the degradation of the ECM and, thereby, may prevent endothelial cell migration and/or, control the activation of growth factors or limit their release from the matrix (40). In this context, TIMP-2 could control the MMP-9 effect on the release of VEGF sequestered in the ECM (41). Alternatively, TIMP-2 may operate by a different mechanism, independently of its antiproteolytic activity. For instance, TIMP-2 may affect directly endothelial cell functions by inhibiting their proliferation or migration (42).

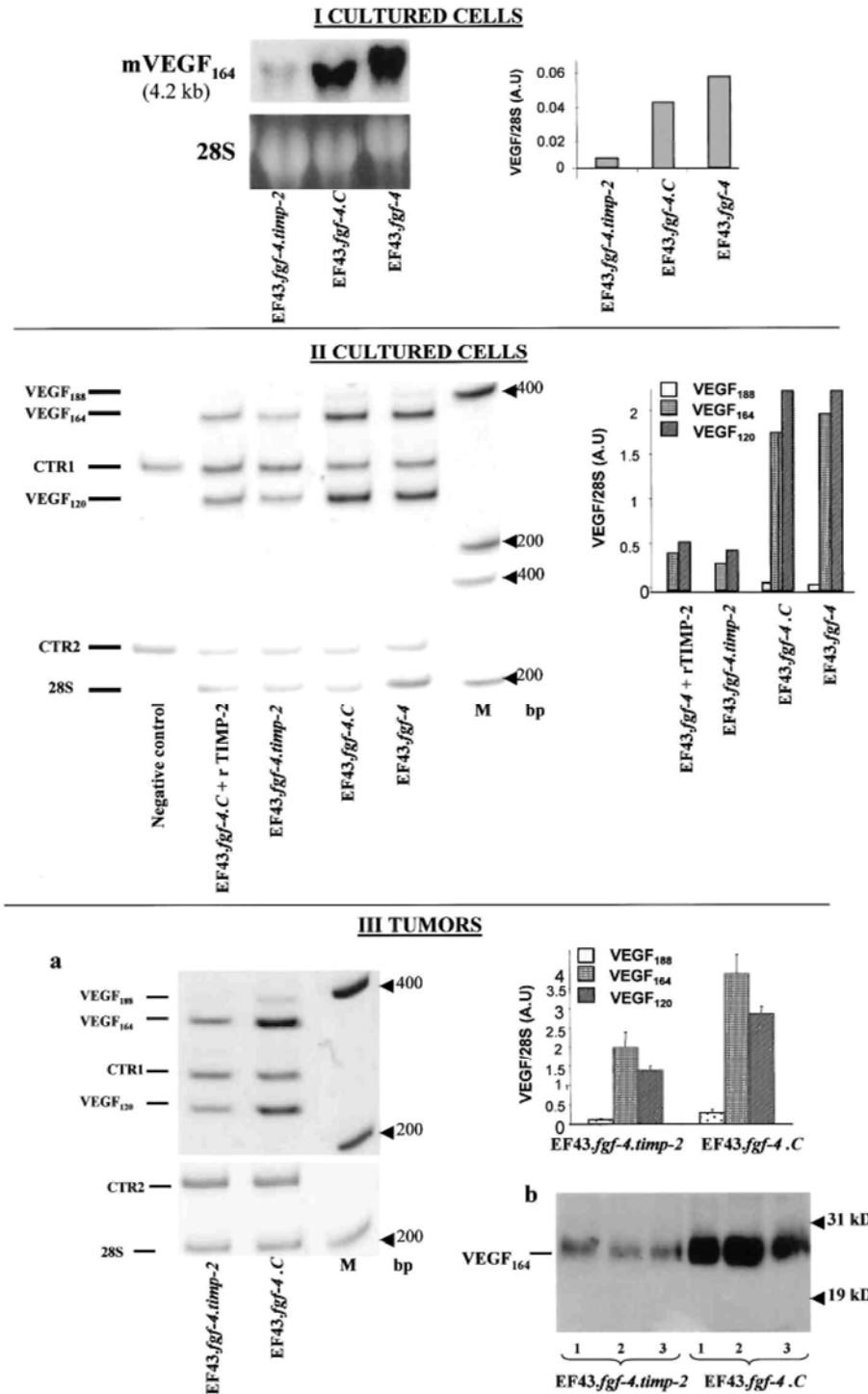
Finally, the antitumoral effect of TIMP-2 was compared with that of angiostatin, which is known for its efficiency on suppressing primary tumor growth (30). Delivery of TIMP-2 or angiostatin was achieved by systemic administration of recombinant defective adenoviruses. In both cases, this resulted in a reduced tumor growth demonstrating that TIMP-2 and angiostatin can have anticancer actions.

Altogether, our data emphasize the importance of TIMP-2 as an anticancer agent. We demonstrate that TIMP-2 reduces tumor growth and angiogenesis and provide a new mechanism for this antitumoral action by reducing dramatically VEGF expression. These data have direct implication in anticancer therapies. They potentially extend the use of TIMP-2 in cancer beyond the control of invasion and metastasis.

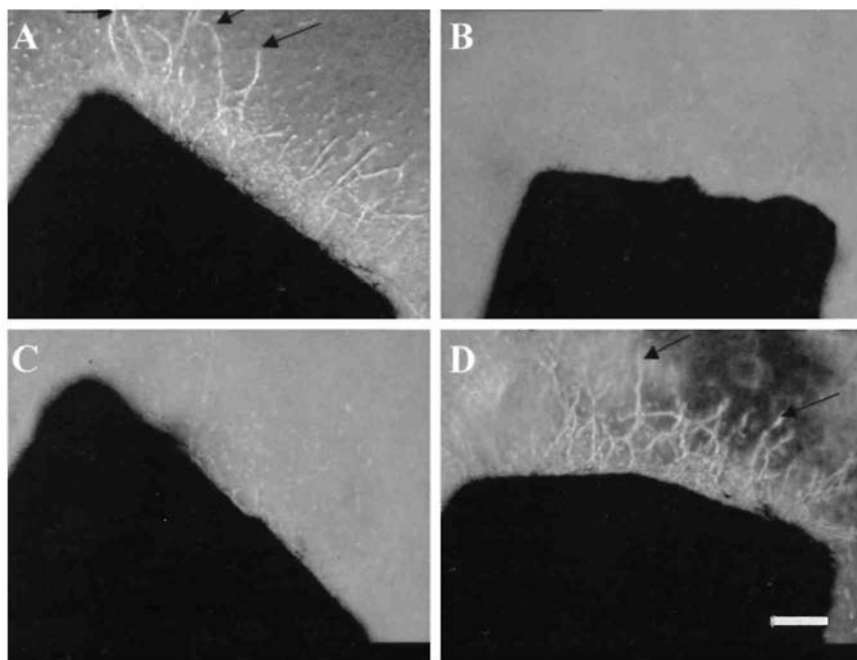
**Fig. 5: Vessel density.** Tumor vascularization was quantified in EF43.fgf-4.C and EF43.fgf-4.timp-2 tumors with defined volumes of 0.4 cm<sup>3</sup> (A), 3.2 cm<sup>3</sup> (B), and 6.2 cm<sup>3</sup> (C). Frozen sections were immunostained with an antilaminin antibody, and tumor vascularization was estimated by image analysis. Results are expressed as mean vessel density  $\pm$  SD (number/mm<sup>2</sup>) as described in "Materials and Methods."



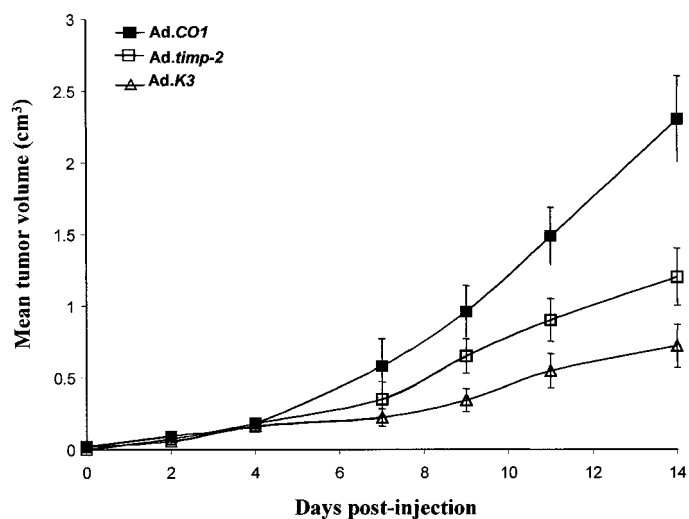
**Fig. 6:** Analysis of VEGF expression. I, Northern blot analysis of *in vitro* cultured cells. Samples, fractionated by gel electrophoresis, were transferred to nylon membrane, verified for gel loading (28S), and hybridized with a probe for mouse VEGF (4.2 kb). The histogram corresponds to the quantification of VEGF mRNA by densitometry. A. U, arbitrary unit. II, quantitative RT-PCR analysis of *in vitro* cultured cells. EF43.fgf-4.C control cells were cultured in the absence or in the presence of rTIMP-2 (EF43.fgf-4.C cells + rTIMP-2). Negative control, no template; M, molecular marker (Life Technologies, Inc.). 28S was used as a control for RNA expression CTR1 and CTR2 correspond to synthetic internal control RNA for VEGF and 28S, respectively. The histogram corresponds to the densitometric quantification of VEGF mRNA isoforms. III,a, quantitative RT-PCR analysis on extracts from tumors with a volume of 3 cm<sup>3</sup>. The histogram corresponds to the densitometric quantification of VEGF mRNA isoforms. III,b, Western blot analysis of tumor extracts. Three different representative tumors (1, 2, 3) derived from EF43.fgf-4. *timp-2* or EF43.fgf-4. C cells were used.



**Fig. 7:** Photomicrographs of rat aorta in collagen gel cultures. Aortic explants were cultured in the presence of media conditioned by EF43.fgf-4.C (A) or EF43.fgf-4.timp-2 cells (B). C, the conditioned medium of EF43.fgf-4 cells was supplemented with rTIMP-2 (100 ng/ml). As positive control, human rVEGF<sub>165</sub> (20ng/ml) was added to the MCDB131 control medium in D. Arrows, microvessels. Bar, 250  $\mu$ m.



**Fig. 8:** In vivo efficiency of adenovirus-mediated gene delivery. Ad.timp-2 or Adk3 (angiostatin) in the amount of  $5 \times 10^9$  pfu was injected i.v. into mice with tumors ( $20 \text{ mm}^3$ ) derived from EF43.fgf-4.C cells. The control AdCO1 vectors were similarly injected into control mice. Each experimental animal group contained seven mice, and the tumor growth was expressed by the tumor mean volume ( $\text{cm}^3$ )  $\pm$  SD



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