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Thymidine phosphorylase is angiogenic and promotes tumor growth

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ABSTRACT **Platelet-derived endothelial cell growth factor was previously identified as the sole angiogenic activity present in platelets; it is now known to be thymidine phosphorylase (TP). The effect of TP on [*methyl*-^{3}H]thymidine uptake does not arise from *de novo* DNA synthesis and the molecule is not a growth factor. Despite this, TP is strongly angiogenic in a rat sponge and freeze-injured skin graft model. Neutralizing antibodies and site-directed mutagenesis confirmed that the enzyme activity of TP is a condition for its angiogenic activity. The level of TP was found to be elevated in human breast tumors compared to normal breast tissue ($P < 0.001$). Overexpression of TP in MCF-7 breast carcinoma cells had no effect on growth *in vitro* but markedly enhanced tumor growth *in vivo*. These data and the correlation of expression in tumors with malignancy identify TP as a target for antitumor strategies.**

Intense interest has recently centered on the angiogenic process, largely as a result of the realization that disregulated blood vessel growth plays a critical role in several disease states, including (*inter alia*) cancer (tumor angiogenesis), diabetic retinopathy, psoriasis, rheumatoid arthritis, and hyperproliferation of the vasa vasorum in atherosclerosis (1). Platelet-derived endothelial cell growth factor was described as a mitogenic and angiogenic factor present in platelets (2). Platelet-derived endothelial cell growth factor is now known to be thymidine phosphorylase (TP) (3) and the effects of TP on cellular uptake of [*methyl*-^{3}H]thymidine when it is added exogenously to cells arise from its effect on the availability of thymidine in the extracellular culture medium (4–6).

Several years prior to the isolation of the so-called platelet-derived endothelial growth factor and demonstration of its angiogenic activity, TP had been identified as an enzyme whose level was significantly elevated in the plasma of cancer patients relative to that in healthy volunteers (7) and in the plasma of xenografted mice relative to that of controls (8) and expressed at a high level in tumors (9, 10). We have found (11) that expression of TP shows a strong correlation with ovarian malignancy and ovarian tumor blood flow.

We report here that TP is angiogenic in the rat sponge model (12) and in a freeze-injured skin graft model (13). Expression of TP strongly correlated with malignancy in breast tumors, and while expression of TP had no effect on breast carcinoma cell growth *in vitro*, it greatly enhanced tumor growth *in vivo*.

MATERIALS AND METHODS

Cell Isolation and Culture. Bovine aortic endothelial cells were isolated as described (14) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum. Low-passage MCF-7 cells (passage 53) were a gift from Marc Lippman (Georgetown University, Washington, DC).

Antibodies to TP. Rabbit anti-TP antisera and mouse anti-TP monoclonal antibodies were raised against recombinant TP expressed in *Escherichia coli* (4).

Site-Directed Mutagenesis and Protein Expression. Three site-directed mutants of TP were constructed by a two-step PCR (15). Recombinant proteins were expressed in *E. coli* using the expression vector pET-14b (ams Biotechnology, Witney, U.K.) and purified by metal chelate affinity chromatography. By gel filtration, recombinant TP was dimeric with a molecular mass of 100 kDa. Concentrations of pure TP were calculated by using a molecular mass of 100 kDa.

Rat Sponge Angiogenesis Assay. Sterile circular polyether sponge discs with central cannula were implanted subcutaneously in male Wistar rats (180–200 g) after induction of neuroleptanalgesia by Hypnorm (12). Four sponges were used in each experimental group. Protein in 50 μl of PBS was injected daily into the sponge. Rabbit anti-TP polyclonal antibody was purified on a protein A-Sepharose column and 25 μg was injected daily into the sponge. Vascularization was assessed as a function of blood flow through the implants by direct injection of ^{133}Xe-containing saline into the sponge and its clearance was monitored over a 6-min period (12). Sponge sections (10 μm) were stained with hematoxylin/eosin, and the fibrovascular growth areas were measured by the axial strip sampling technique (16) that uses computer-assisted image analysis. The length/density ratio of blood vessels was calculated as described (17).

Rat Freeze-Injured Skin Graft Assay. Isolated rat skin explants were treated with the cryoprotectant dimethyl sulfoxide [25% (vol/vol) in medium 199] and cooled to −196°C prior to rapid warming and grafting back into the skin as described (13). TP (500 ng) was incorporated into 10 slow-release Elvax pellets that were implanted around the perimeter of the graft.

Endothelial Cell Migration. The substance under test was placed into the bottom well of a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, MD). Subconfluent bovine aortic endothelial cells were removed by exposure to trypsin and seeded (20,000 cells per well) onto a 8-μm polyvinylpyrrolidone membrane (Costar) that had been coated for 4 h with a solution of fibronectin (100 ng/ml) in PBS. After incubation for 5 h at 37°C in 5% CO_2/95% air, membranes were disassembled and fixed in methanol (5 min). Cells were stained with hematoxylin and three fields were counted per well at ×400 with a graticule (18).

Western Blot Analysis. Human breast tissue cytosol was prepared as described (19). Total cytosol protein (80 μg) was

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Abbreviations: TP, thymidine phosphorylase; WT, wild type.

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analyzed by SDS/PAGE and electroblotted onto an Immobilon-P membrane (Millipore). TP was visualized with rabbit polyclonal anti-TP antisera and ^{125}I-labeled protein A.

Immunocytochemistry. Paraffin-embedded human breast tissue sections were immunostained with anti-TP monoclonal antibody (P-GF.44c). Antibodies were visualized by the streptavidin-biotin-peroxidase technique (20).

Overexpression of TP and Breast Carcinoma Growth. The TP gene under control of the cytomegalovirus promoter in plasmid pcDNA-1-neo (Invitrogen), was transfected into MCF-7 breast carcinoma cells and clones were isolated as described (21). Xenografts of the MCF-7 transfectants in ovariectomized BALB/c *nu/nu* mice were performed as described (21).

RESULTS

Angiogenic Activity of TP. Angiogenic activity of TP was determined by using the rat sponge model. Fig. 1*A* shows that recombinant human TP expressed in *E. coli* is angiogenic in the rat subcutaneous sponge model (12). A statistically significant ($P < 0.001$) enhancement of angiogenesis was observed when daily doses of 10 or 100 pmol of TP were administered into the sponge but not with doses of 0.1 or 1 pmol ($P < 0.1$). Fig. 1*B* shows that coadministration of polyclonal rabbit anti-TP antibodies into the sponge completely blocked TP-stimulated angiogenesis, whereas the antibodies had no effect on angiogenesis induced by basic fibroblast growth factor.

TP was also examined for angiogenic activity in a freeze-injured skin graft assay. We have shown (13) that revascularization of full-thickness skin autografts in rats is delayed by subjecting the graft to a sublethal freeze injury. Graft blood flow was determined by both laser Doppler flowmetry and the rate of ^{133}Xe clearance. Fig. 1 shows that there was a statistically significant stimulation of graft blood flow with the application of 500 ng of TP per graft, when blood flow was quantitated by laser Doppler flowmetry (Fig. 1*C*) or by ^{133}Xe clearance (Fig. 1*D*). Fig. 2 shows TP induced vascularization compared to the control. Analysis of the sponges by measurements of the fraction of volume of fibrovascular growth and the length/density ratio of blood vessels confirmed that vascularization in the TP-treated sponges was significantly greater than in the control sponges (Fig. 2 *C* and *D*).

Angiogenic Activity of TP Mutants. To determine whether the enzyme activity of TP is required for angiogenic activity, active site mutants of human TP were prepared by site-directed mutagenesis. The mutations, namely, Arg-202 → Glu, Lys-221 → Glu, and Ser-217 → Ala, corresponded to residues in the *E. coli* TP that had been shown by x-ray crystallography to bind the nucleoside (22, 23). The Arg and Lys mutants were enzymatically inactive [$<10^{-4}$ of the wild type (WT)], whereas the Ser mutant retained 1.3% of the activity of WT/TP when assayed with thymidine. Like WT/TP, all three mutants spontaneously dimerized, as judged by gel filtration (data not shown), and we surmise that they retained an overall structure similar to that of WT/TP. Fig. 3 shows that all three TP mutants were angiogenically inactive when administered in daily doses of 10 or 500 pmol.

Chemotactic Activity of TP and Its Substrates/Products. TP stimulated endothelial migration at 10 and 100 ng/ml in the

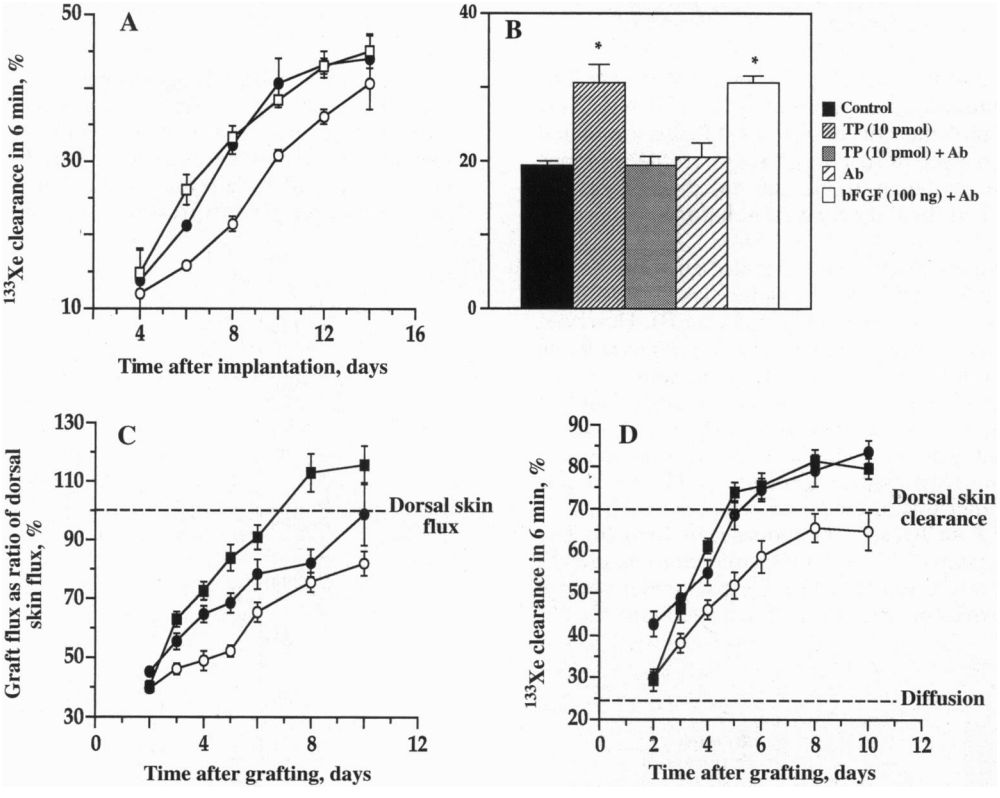

FIG. 1. Angiogenic activity of TP in the rat sponge and freeze-injured skin graft models. (*A*) Dose–response curves of the angiogenic activity of recombinant human TP (○, PBS control; ●, 10 pmol of TP per day; □, 100 pmol of TP per day) in the rat sponge model ($n = 4$, mean ± SEM). A daily dose of 0.1 or 1 pmol of TP gave no significant stimulation above controls. (*B*) Blocking of TP-induced sponge angiogenesis by polyclonal rabbit anti-TP antibodies (Ab). Polyclonal anti-TP antibodies failed to block basic fibroblast growth factor (bFGF)-induced angiogenesis ($n = 4$, mean ± SEM). (*C* and *D*) Angiogenic activity of human recombinant TP in the freeze-injured skin graft model. Graft blood flow was measured by laser Doppler flowmetry and expressed as a percentage of that determined for the uninjured skin of the dorsal flank (*C*) and by monitoring the rate of ^{133}Xe clearance and expressed as the percentage of clearance over 6 min (*D*). ○, Control; ●, TP containing pellets; ■, non-freeze-injured graft. Data are the mean ± SEM ($n = 5$ to 38). Some animals were sacrificed for histology on days when measurements were made; thus, the number of data points fell as the experiment progressed.

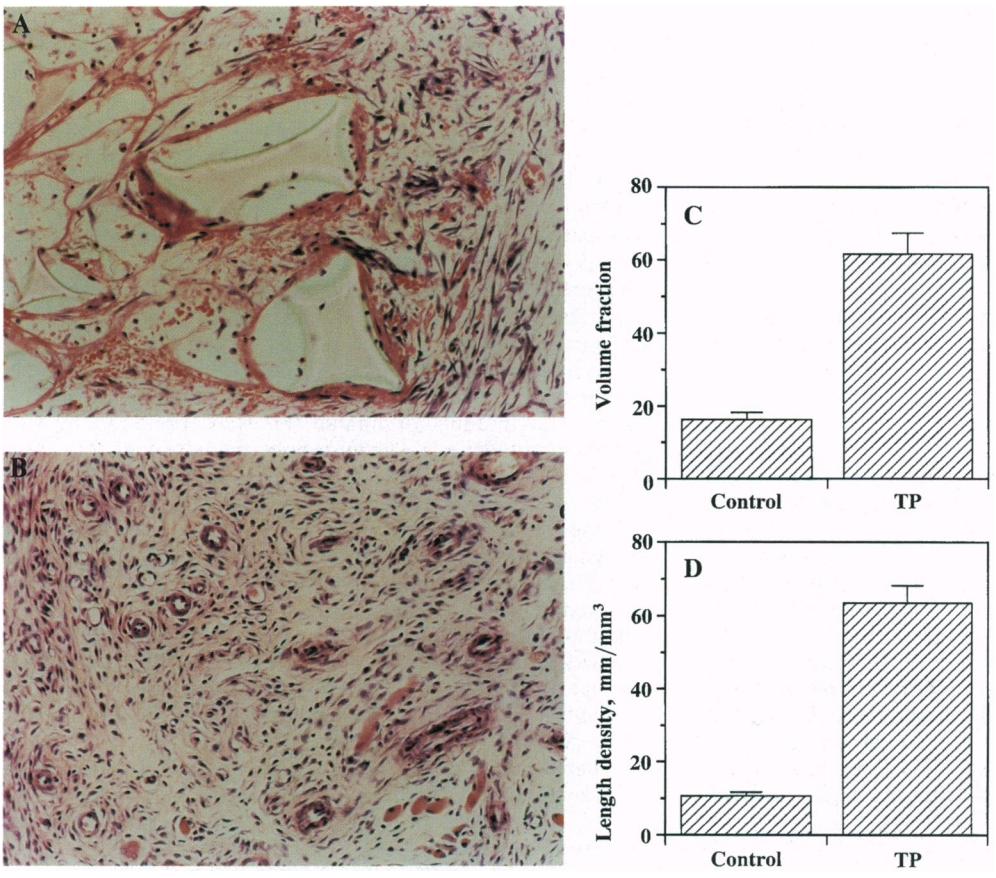

FIG. 2. Histology of control and TP-treated sponge implants from day 8. Hematoxylin/eosin-stained sections of control (saline) (*A*) and recombinant human TP-treated (*B*) sponges on day 8. (*C* and *D*) Effect of TP on the fractional volume of fibrovascular growth into the sponge (*C*) and the length/density ratio of blood vessels in the sponge implants (*D*) ($n = 4$; $P < 0.001$).

presence of 1% fetal calf serum (Fig. 4*A*) as described (2). The three active site mutants were without activity in this assay when examined at the same concentrations. TP also stimulated migration in the absence of serum but required the presence of 0.1% bovine serum albumin and 100 nM thymidine (Fig. 4*B*). In the absence of thymidine, no stimulation of migration was detected.

TP in Breast Tumor Tissue. Western blot analysis showed that TP is overexpressed in breast carcinomas compared to normal breast tissue ($P < 0.001$) (Fig. 5 *A* and *B*). However, immunohistochemistry revealed that the cell type expressing TP varied between tumors (Fig. 5*C*). Thus, in some tumors, expression was restricted to the neoplastic element and in others it was restricted to the stromal or inflammatory component. Some staining of tumor capillary endothelium was also evident. In normal breast tissue, expression of TP was largely restricted to the inner ductal epithelial cells (Fig. 5*C*).

The Effect of TP on Breast Carcinoma Cell Growth. The effect of overexpression of TP on breast carcinoma cell growth *in vitro* and *in vivo* was examined. The TP gene under control of the cytomegalovirus promoter was transfected into MCF-7 breast carcinoma cells. Xenografts were performed twice with three overexpressing clones; the data for clone TP-7 and control cells transfected with the empty vector plasmid are shown in Fig. 6. Overexpression of TP in the transfected cells was characterized by enzyme activity in cell lysates and is shown in Fig. 6*A*. Overexpression of TP had no effect on *in*

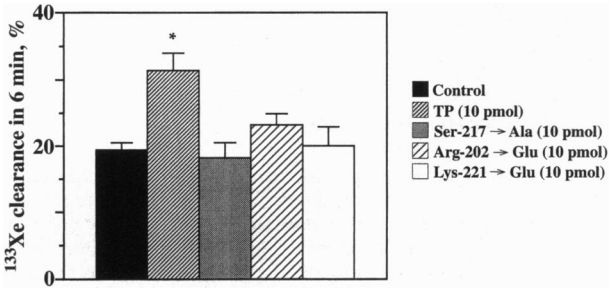

FIG. 3. Angiogenic activity of WT TP and site-directed TP mutants. A statistically significant enhancement of the rate of ^{133}Xe clearance was seen only on administration of WT TP. *, $P < 0.01$ vs. control.

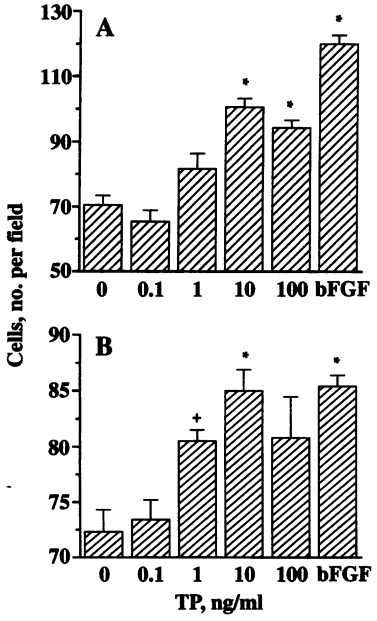

FIG. 4. Migration of bovine aortic endothelial cells in a modified Boyden chamber. (*A*) In DMEM supplemented with 1% fetal calf serum. (*B*) In DMEM supplemented with 0.1% bovine serum albumin and 100 nM thymidine. Data are the mean ± SEM ($n = 4$). *, $P < 0.001$; +, $P < 0.02$. Each experiment was repeated three times with similar results. bFGF, basic fibroblast growth factor.

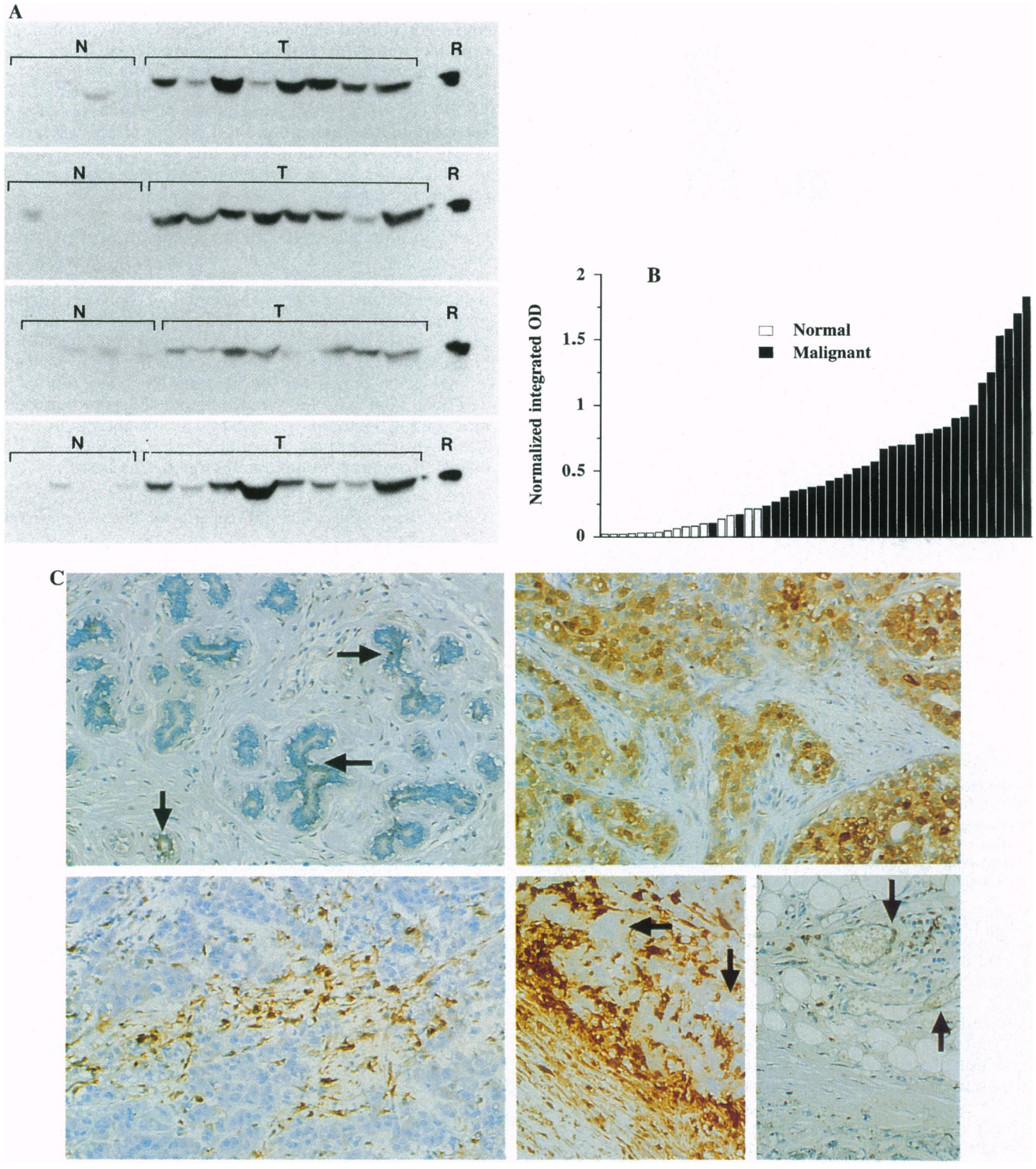

FIG. 5. Expression of TP in normal human breast tissue and breast carcinomas. (*A*) Western blot analysis of various normal and malignant breast tissues. N, normal; T, tumor; R, recombinant TP. (*B*) Densitometric quantitation of the Western blot analysis shown in *A*. Sixteen normal breast tissues and 32 malignant tumors were examined. (*C*) Immunohistochemical staining of TP in normal and malignant breast tissues. (*Upper Left*) Normal breast tissue. The inner ductal epithelial cells are positive (arrows), whereas the myoepithelial cells are negative. (*Upper Right*) Invasive ductal carcinoma that shows positive staining of the tumor cells with negative stroma. (*Lower Left* and *Middle*) Invasive ductal carcinomas that show positive staining of the inflammatory and stromal cells, respectively, with negative neoplastic cells (arrows). (*Lower Right*) Tumor vessels that show positive staining endothelium (arrows).

vitro growth (Fig. 6*B*) but all overexpressing TP clones gave statistically significantly faster growing tumors than did control cells (Fig. 6*C*).

DISCUSSION

The results presented here show that recombinant TP induces vascularization in the rat sponge model and in the rat freeze-injured skin graft model. This is consistent with the reported activity of TP in the chicken chorioallantoic membrane (2). That the enzyme activity is required for angiogenic activity was shown with three active site mutants that maintained their secondary structure, as determined by their capacity to dimerize, but had reduced TP activity. The mutants did not exhibit angiogenic activity in the rat sponge model when assayed at the

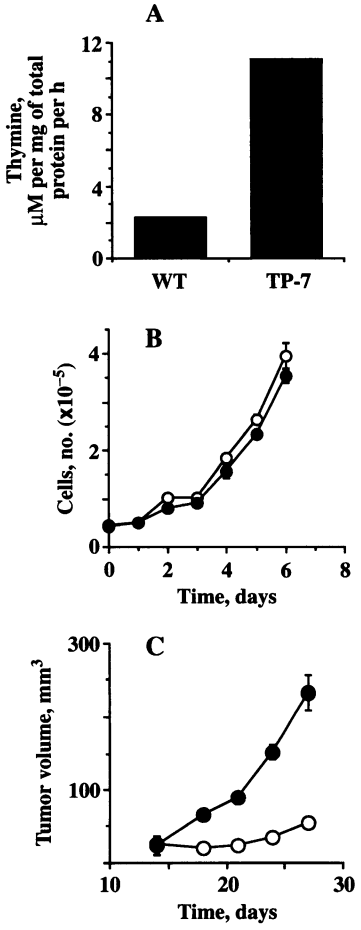

FIG. 6. Effect of overexpression of TP on MCF-7 breast carcinoma growth. (*A*) TP activity of WT cells (transfected with vector alone) and TP-transfected (TP-7) cells. (*B*) Growth of WT (○) and TP-7 (●) cells in 1% fetal calf serum *in vitro*. (*C*) Growth of WT (○) and TP-7 (●) cells when xenografted into BALB/c *nu/nu* mice (n = 10; average ± SD). Xenografts were performed twice with three overexpressing clones. All overexpressing TP clones gave statistically significant faster growing tumors than did WT cells. Representative data for TP-7 are shown.

same dose or at 50 times the dose of the WT/TP. There was a good correlation between the angiogenic activity of TP *in vivo* and its chemotactic activity for endothelial cells *in vitro*. Preliminary experiments have shown that the Ser-217 → Ala mutant, which has 1.3% of the enzyme activity of WT/TP, stimulated migration of endothelial cells *in vitro* at a dose 1000 times (but not 100 times) that of WT/TP (data not shown). TP stimulation of endothelial cell migration *in vitro* only occurred in the presence of thymidine. We conclude that despite the fact that TP is not a growth factor for endothelial cells, it is angiogenic *in vivo*. The angiogenic activity requires the enzyme activity and may be the result of its chemotactic activity for endothelial cells.

Western blot analysis of human breast tissue showed that the level of TP protein was elevated in tumors compared to normal tissues. Similar findings have been reported for several other solid tumor types but the reverse for hyperproliferative diseases of lymphocyte origin. It is in solid tumors that an angiogenic stimulus might be expected to confer a growth advantage. Expression of TP in human breast tissue was variable in cellular origin, thus the carcinoma, inflammatory cells, the stromal cells, or all overexpressed TP. To a lesser extent, staining of capillary endothelium was also seen. While it is not known what is regulating TP expression in breast tumors, TP is known to be induced in several carcinoma lines (but not in normal fibroblasts) within 6 h by the inflammatory cytokines tumor necrosis factor α, interleukin 1, and interferon γ and induced up to 47-fold by a mixture of all three (24). Transfection experiments showed that overexpression of TP in MCF-7 cells conferred a growth advantage to these cells when xenografted into nude mice but it did not affect their growth *in vitro*. The growth enhancement *in vivo* may arise as a result of transfection of an angiogenic advantage although this remains to be proven.

Some nucleotide metabolites, for example, adenosine, are known to stimulate proliferation and migration of endothelial cells *in vitro* (25) and to be angiogenic *in vivo* (26). TP hydrolyzes thymidine to 2′-deoxy-D-ribose 1-phosphate and thymine. In turn, 2′-deoxy-D-ribose 1-phosphate is dephosphorylated to 2′-deoxy-D-ribose, which has recently been reported to be angiogenic in the chicken-chorioallantoic membrane assay (27). However, the exact mechanism by which TP is angiogenic remains to be proven. The properties of TP described here raise the interesting possibility of therapeutic intervention in tumor growth by specific inhibition of TP. Although at present no particularly potent inhibitors of TP are known, 6-amino-5-bromouracil is among the best (28).

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