Antitumor Activities of TEM8-Fc: An Engineered Antibody-like Molecule Targeting Tumor Endothelial Marker 8

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Tumor endothelial marker 8 (TEM8) was discovered as a cell membrane protein that is predominantly expressed in tumor endothelium and identified as a receptor for anthrax toxin. We developed an antibody-like molecule that consists of the protective antigen (PA)–binding domain of human TEM8 linked to the Fc portion of human immunoglobulin G1 (TEM8-Fc). This engineered protein bound to PA in a divalent cation–dependent manner and efficiently protected J774A.1 macrophage-like cells against anthrax toxin challenge in a dose-dependent manner. TEM8-Fc suppressed the growth and metastasis of xenograft human tumors in athymic nude mice (control versus 10 mg/kg TEM8-Fc, mean tumor weight: LS-180, 1.72 versus 0.16 g, difference = 1.56 g, 95% confidence interval [CI] = 0.96 to 2.16 g; *P*<.001; MCF-7, 1.12 versus 0.08 g, difference = 1.04 g, 95% CI = 0.77 to 1.31 g; *P*<.001; HepG2, 1.28 versus 0.35 g, difference = 0.93 g, 95% CI = 0.60 to 1.25 g; *P*<.001). Furthermore, TEM8 interacted with the M2 isoenzyme of pyruvate kinase (M2-PK), which has an important role in tumor growth and metastasis. TEM8-Fc is a novel therapeutic antibody-like agent in the management of solid tumors that may act by trapping M2-PK.

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It has been well established that uncontrolled angiogenesis contributes greatly to the malignant growth and metastasis of primary tumors. In addition, inhibition of tumor angiogenesis as an attractive anticancer strategy has gained widespread support from cancer researchers and clinicians (1–3). This anticancer strategy has several theoretic advantages (4–6), including broad-spectrum antitumor activities, lower incidence of drug resistance, and the avoidance of pharmacokinetic problems.

Vascular endothelial growth factor (VEGF) and its receptor (VEGFR) are expressed by the activated endothelial cells of newly formed blood vessels and play a key role in tumor angiogenesis (7–9). At present, several compounds inhibit the activation of VEGFR and have potent antitumor activity. In addition, a humanized monoclonal anti-VEGF antibody (Avastin, Genentech) has been developed to block VEGF signaling, and it effectively inhibits tumor growth (10). However, VEGF and VEGFR are not exclusively expressed in tumor endothelium, which limits the application of these antiangiogenic agents. For this reason, a better understanding of the molecular differences between normal and tumor vessels is required to realize the full potential of antiangiogenic approaches.

Recently, a new class of molecules named tumor endothelial markers (TEM1–9) has been identified from comparisons of global gene expression patterns in human endothelial cells of normal and malignant colorectal tissues (11). These molecules appear to be expressed specifically in tumor endothelium and are potentially involved in tumor angiogenesis. Among them, TEM8 was especially interesting because of its cell-surface localization and high amino acid sequence conservation across species. Furthermore, TEM8 is the only tumor endothelial marker characterized to date that is not expressed in either the corpus luteum or healing wounds, suggesting that it is highly specific to tumor angiogenesis and not required for normal adult angiogenesis (11,12). Interestingly, TEM8 was also identified as a receptor for protective antigen (PA), the cell-binding component of anthrax toxin, which prevents the toxin from entering cells (13). The extracellular region of TEM8 contains a von Willebrand factor type A domain, which is often found in the extracellular domains of integrins (14). The cytoplasmic tail of TEM8 is much larger than that of other cell-surface tumor endothelial markers and contains at least seven potential phosphorylation sites, supporting the hypothesis that TEM8 is involved in signaling pathways that regulate tumor-specific angiogenesis (12). Two reports (15,16) have also indicated that TEM8 plays a positive role in endothelial cell activities related to angiogenesis, such as migration, adhesion, and tube formation. Taken together, all of these characteristics make TEM8 particularly useful in the development of neovascularization-targeted antitumor agents.

Bearing this in mind and by learning of the successful development of VEGF-Trap (17), Enbrel (tumor necrosis factor-α receptor–Fc fusion protein) (18), and Pro-542 (CD4-immunoglobulin G2 [IgG2]) (19), we developed an antibody-like molecule (i.e., TEM8-Fc fusion protein) that consists of the N-terminal 200 amino acid residues (without the leader peptide 1–27 amino acid residues) of human TEM8 linked to the 232 amino acid residues from the Fc portion (hinge, CH2, and CH3 domains) of human IgG1 (Fig. 1, A–C). This engineered protein bound to PA in a divalent cation–dependent manner similar

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CONTEXT AND CAVEATS

Prior knowledge

Tumor endothelial marker 8 (TEM8) is an anthrax toxin receptor that is expressed on the plasma membrane of tumor endothelial cells.

Study design

TEM8-Fc, an antibody-like molecule that contains the protective antigen (PA) domain of TEM8 fused to the Fc portion of human immunoglobulin G1 was synthesized, and PA functions were assayed. Tumor growth and metastasis were compared in TEM8-Fc–treated and control athymic mice carrying xenograft tumors derived from human cell lines. Immunoprecipitation with TEM8-Fc was performed using tumor homogenates.

Contributions

TEM8-Fc retained properties of the PA-binding domain and reduced tumor growth and metastasis in the mice carrying xenograft tumors. TEM8-Fc bound to the M2 isoenzyme of pyruvate kinase (M2-PK), which is involved in tumor growth and metastasis.

Implications

TEM8-Fc is an antibody-like molecule that may suppress tumor growth and metastasis by trapping the M2-PK.

Limitations

It is unknown whether TEM8-Fc would be a useful therapeutic agent in human cancer.

to TEM8, as previously described (20) (Fig. 1, D) and could efficiently protect a macrophage-like cell line, J774A.1 cells (American Type Culture Collection [ATCC], Rockville, MD), against anthrax toxin challenge (Fig. 1, E). These results indicate that the recombinant TEM8-Fc retains the ability to bind to PA and function like soluble TEM8 in that context.

To examine the broad-spectrum antitumor activities of TEM8-Fc, we chose three cell lines (LS-180, MCF-7, and HepG2 cells; ATCC) that were derived from cancers of the colon, breast, and liver, respectively, all of which have high incidence and/or high mortality rates. Treatment with TEM8-Fc markedly inhibited the growth of xenograft tumors that were derived from these cell lines. In addition, the control IgG had no effect on tumor growth (Fig. 2, A). These results indicate that the antitumor effects of TEM8-Fc are

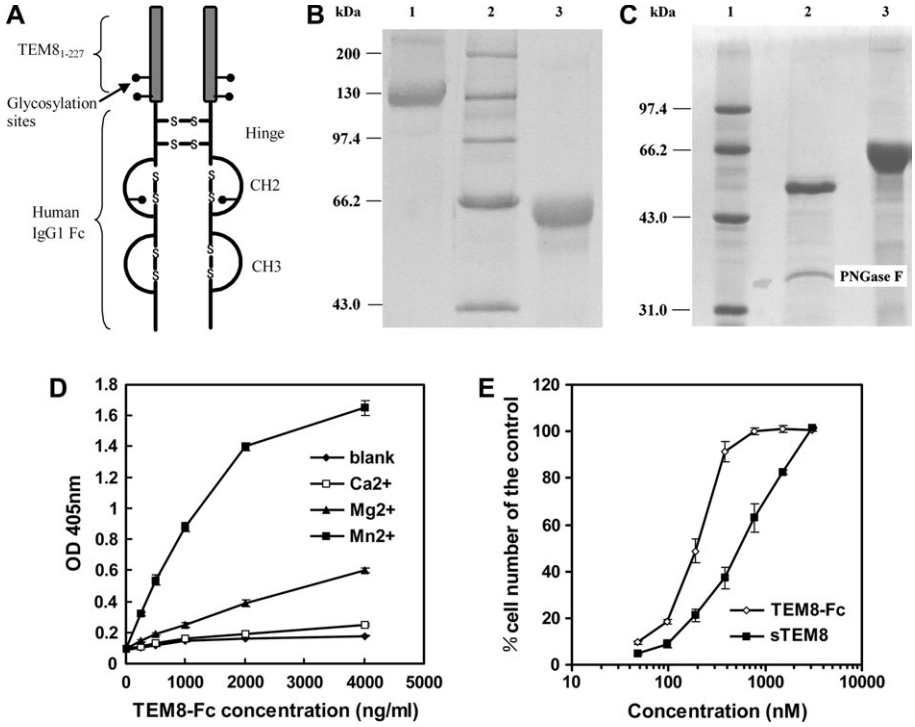

Fig. 1. Expression, purification, and characterization of the fusion protein of the N-terminal 1-227 amino acid residues of human tumor endothelial marker 8 (TEM8) linked to the Fc portion of human immunoglobulin G1 (IgG1) (TEM8-Fc). Chinese hamster ovary (CHO) cells were transfected with pcDNA3.1/TEM8-Fc using Lipofectamine 2000 reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. The transfected cells were selected by G418, and the cells highly expressing recombinant TEM8-Fc were identified by direct enzyme-linked immunosorbent assay (ELISA). As previously described (27), a recombinant CHO cell line, TEM8-Fc-1D5, which expresses TEM8-Fc at the level of 10–15 pg/cell per day, was chosen and cultivated in roller bottles with Dulbecco's modified Eagle Medium/F12 (1:1) (Hyclone, Logan, UT) supplemented with 2% Cosmic Calf serum (Hyclone) until the cells reached 90% confluency. Cells were then grown in serum-free medium developed by our laboratory (28), which was replaced every 2 days. The conditioned media were collected at each change of media, and supernatant was purified by means of a protein A-Sepharose 4 Fast Flow affinity chromatography (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. TEM8-Fc was eluted with 0.1 M glycine buffer, pH 2.5, and the pH of the collected fraction was immediately adjusted to 7.0 by the addition of 1 M Tris–HCl buffer, pH 9.0. TEM8-Fc was concentrated and desalted by ultrafiltration using Centriplus Centrifugal Filter Devices (50000 MWCO, Millipore Corporation, Bedford, MA) and lyophilized. Before use, TEM8-Fc was rehydrated with saline (0.9%) and filtration-sterilized. The purified protein was analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and stained with Coomassie blue under nonreducing or reducing conditions to estimate the molecular weight and verify the purity and homogeneity. To analyze the glycosylation of TEM8-Fc, the fusion protein was treated with PNGase F (New England Biolabs, Beverly, MA) to remove the carbohydrate residues. In brief, 20 μg of TEM8-Fc was denatured in 20 μL denaturing buffer (0.5% SDS, 1% 2-mercaptoethanol) at 100 °C for 10 minutes. Then 2 μL of 10× reaction buffer (500 mM sodium phosphate, pH 7.5, 10% NP40) and 200 units of PNGase F were added. After incubation at 37 °C for 1 hour, the sample was analyzed by 12% SDS–PAGE under reducing conditions. **A**) Schematic diagram of homodimeric TEM8-Fc. CH, constant domain of IgG heavy chain. **B**) SDS–PAGE analysis of TEM8-Fc. Lane 1, nonreducing conditions; lane 2, protein marker; lane 3, reducing conditions. **C**) Glycosylation analysis of TEM8-Fc with reducing SDS–PAGE electrophoresis. Lane 1, protein marker; lane 2, deglycosylated TEM8-Fc; lane 3, glycosylated TEM8-Fc. **D**) TEM8-Fc binding with protective antigen (PA) determined by ELISA in the absence or presence of 2 mM $MnCl_2$, $CaCl_2$, or $MgCl_2$ as described previously (20). **E**) Protection of J774A.1 cells against anthrax toxin challenge by TEM8-Fc. Recombinant PA (100 ng/mL) and lethal factor (100 ng/mL) were preincubated with a range of dilutions of TEM8-Fc or soluble TEM8, the extracellular 1–227 amino acid residues of TEM8 expressed by CHO cells, for 30 minutes at 37 °C in a working volume of 100 μL modified Eagle medium (MEM, Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum. This 100-μL volume was subsequently transferred into a 96-well flat bottom tissue culture plate containing 1.0×10^5 J774A.1 cells per well in 100 μL of the same medium. The culture plate was incubated for 3 hours at 37 °C. The number of viable cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St Louis, MO) in which MTT was dissolved in the medium and added to each well at a final concentration of 0.5 mg/mL. Cells were incubated for another 30 minutes at 37 °C. The medium was replaced by 0.5% (wt/vol) SDS and 25 mM HCl in 90% isopropyl alcohol, the plate was vortexed, and the absorbance readings at 570 nm were measured with a microplate reader (Bio-Rad Model 550, Hercules, CA). All experiments were performed in triplicate. In **D** and **E**, **points** represent means and **error bars** 95% confidence intervals.

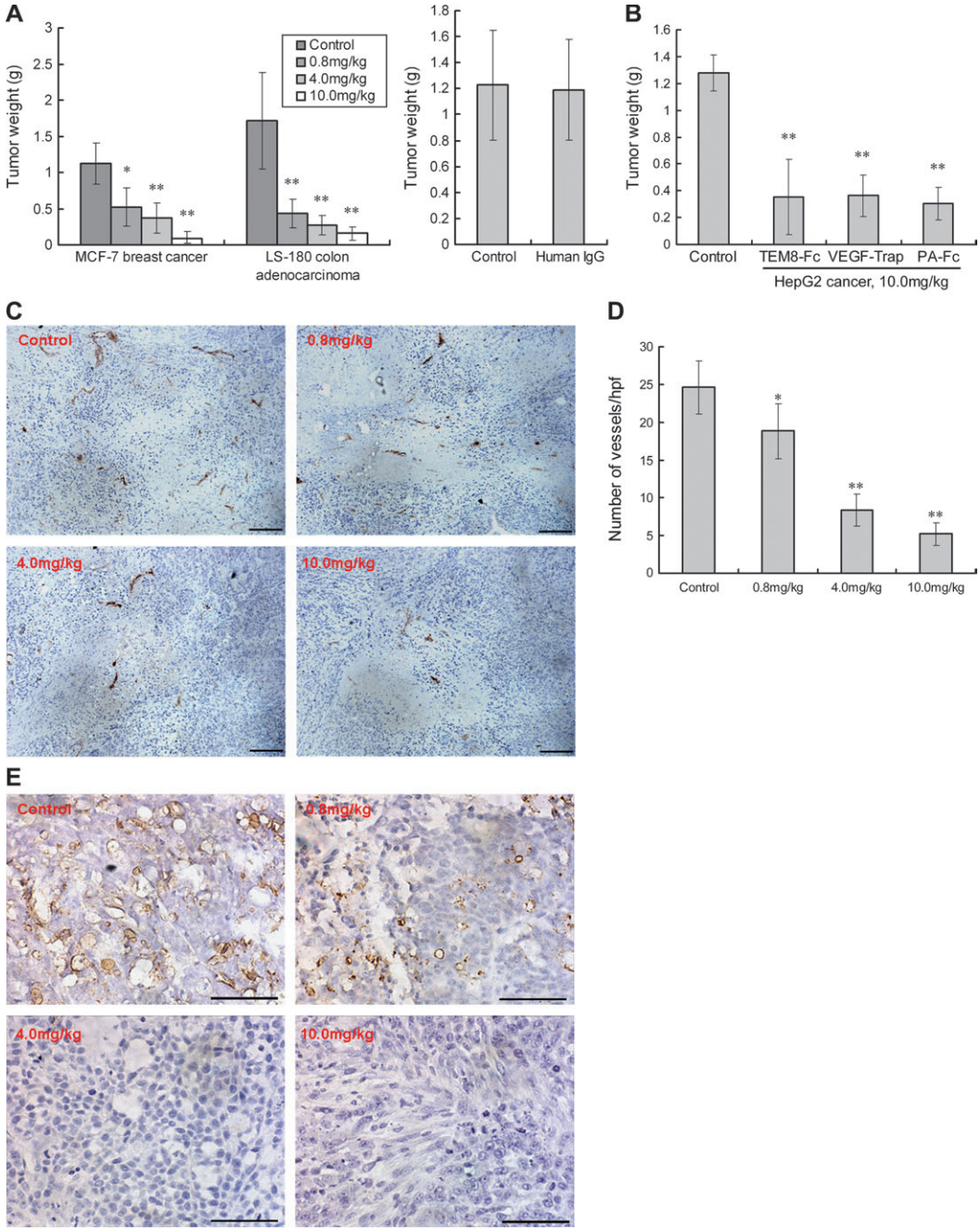

Fig. 2. Effects of the fusion protein of the N-terminal 1-227 amino acid residues of human tumor endothelial marker 8 (TEM8) linked to the Fc portion of human immunoglobulin G1 (IgG1) (TEM8-Fc) on the growth, neovascularity, and malignant phenotype of implanted tumors from diverse tissues. Human tumor cell lines LS-180 (1.0 $\times 10^6$ cells per mouse), HepG2 (3.0 $\times 10^6$ cells per mouse), and MCF-7 (1.5 $\times 10^6$ cells per mouse) were suspended in serum-free media and implanted subcutaneously on the right flank of male athymic nude mice (n = 7), 6–8 weeks old, obtained from the Institute of Experimental Animals, Chinese Academy of Medical Sciences. All mouse protocols were approved by Beijing Institute of Radiation Medicine (IRB) at the indicated concentrations. Mice (n = 7 for each group) were injected subcutaneously with TEM8-Fc (0.8, 4.0, and 10.0 mg/kg), VEGF-Trap (10.0 mg/kg), PA-Fc (10.0 mg/kg), or human IgG (10.0 mg/kg) every 2 days, beginning on the second day of tumor cell implantation. PA-Fc hybrid protein contains the PA domain 4 (29) with the 232 amino acid residues from Fc portion of human IgG1 and was prepared as that of TEM8-Fc. Human IgG proteins were purified from 200 mL of serum of healthy donors (purchased from Affiliated Hospital, Academy of Military Medical Sciences; approved by IRB) mixed with the culture supernatant of nontransfected Chinese hamster ovary cells using the same methods as those used for TEM8-Fc. Control mice were treated with vehicle (0.9% NaCl saline). After 3 weeks [this time was chosen according to Holash et al. (17)], mice were killed and tumors were excised and weighed. For histologic examination, tumors were fixed and embedded in paraffin blocks and then cut into thin sections. The tissue sections were either immunostained with antibody against CD31 or carcinoembryonic antigen. Briefly, paraffin sections were deparaffinized, incubated in citrate buffer, pH 6.0, at 95 °C for 20 minutes, and treated with peroxidase blocking reagent (DAKO, Carpinteria, CA). Sections were incubated with rat monoclonal anti-CD31 (BD Pharmingen, San Jose, CA, 1:20) or rabbit polyclonal anti-CEA antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, 1:50) followed by a horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnologies, 1:1000) and then by diaminobenzidine (Sigma, St Louis, MO) staining. **A**) Effect of TEM8-Fc on the growth of MCF-7 and LS-180 xenograft tumors. **B**) Effect of TEM8-Fc on the growth of HepG2 xenograft tumor growth compared with VEGF-Trap and PA-Fc. Analysis of variance was applied to determine statistical significance of the differences between the various treatment groups and the control group. All *P* values were two-sided. Data are shown as means and 95% confidence intervals. **P* = .0011, ***P*<.001. **C**) Representative photographs of immunohistochemical staining of endothelial marker CD31 in MCF-7 breast tumors (from **A**). **Bar** = 100 μm. **D**) Quantification of blood vessels in MCF-7 xenograft tumors in (**A**) was carried out as previously described (30). The three most vascular areas of tumors were identified on a low-power field in each section, and vessels were counted in high-power fields. Any brown-stained endothelial cell cluster distinct from adjacent tumor cells, or other stromal cells, was considered as a single countable microvessel. The number of microvessels in each group was averaged and expressed as the number per high-power field. The two-sided Mann–Whitney *U* test was applied to determine statistical significance of the differences between the various treatment groups (n = 5 in each experimental group) and the control group (n = 5). Data are shown as means and 95% confidence intervals. **P* = .0468, ***P*<.001. **E**) Representative photographs of LS-180 tumor sections stained with rabbit polyclonal anti-CEA antibody (1:50). **Bar** = 100 μm.

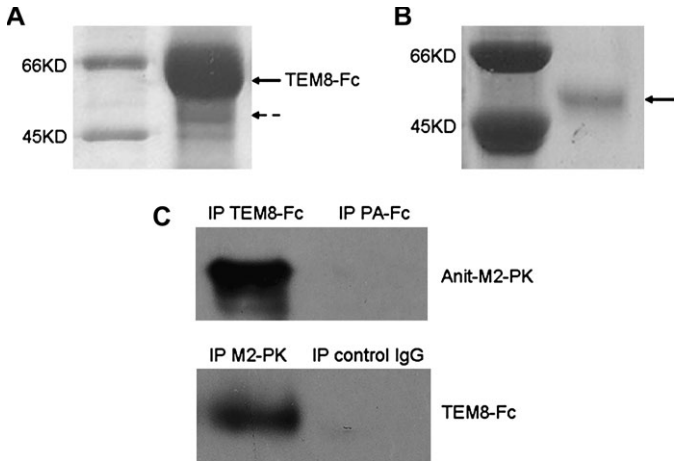

Fig. 3. Identification of the natural interacting partner for tumor endothelial marker 8 (TEM8). The homogenate of HepG2 tumors was pretreated with a protein A-Sepharose 4 Fast Flow affinity column to remove immunoglobulin G (IgG) proteins and was then applied to a protein A-Sepharose 4 Fast Flow affinity column preconjugated with the fusion protein of the N-terminal 1-227 amino acid residues of human TEM8 linked to the Fc portion of human IgG1 (TEM8-Fc). Bound proteins were eluted using the same methods as those used for TEM8-Fc. Then the bound proteins were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and visualized by Coomassie staining. **A**) A distinct protein band was trapped by TEM8 affinity chromatography (**dotted arrow**). This band was excised from the gel, incubated with 10 μg/mL trypsin, and then analyzed by nanoLC-MS/MS using a CapLC liquid chromatography (Waters, Milford, MA) coupled with a Q-TOF Ultima hybrid quadrupile time-of-flight mass spectrometer (Waters). MS/MS fragment ion spectra were searched against the National Center for Biotechnology Information nr protein sequence database using the MASCOT database search engine (Matrix Science, London, UK). **B**) HepG2 tumor homogenates were incubated with 2 μg of rabbit anti–M2-PK polyclonal antibody (Beijing BIOS Biotechnology, Beijing, China) for 2 hours at 4 °C and then with 20 μL of Streamline rProtein A (GE Healthcare, Uppsala, Sweden) overnight at 4 °C. Immunoprecipitates were separated by 12% SDS–PAGE. **C**) Immunoprecipitation (IP) and immunoblotting were performed to verify the interaction between TEM8 and M2-PK. Upper panel: the lysate of HepG2 cells (500 μg) was incubated with 20 μg of TEM8-Fc or PA-Fc for 2 hours at 4 °C and then with 20 μL of Streamline rProtein A overnight at 4 °C. Immunoprecipitates were resolved by 12% SDS–PAGE, followed by immunoblotting with the anti–M2-PK antibody. Lower panel: the lysate of HepG2 cells (500 μg) was immunoprecipitated with 2 μg of anti–M2-PK antibody or isotype-matched IgG (Santa Cruz Biotechnologies), followed by immunoblotting with TEM8-Fc.

specific, not a result of endotoxin or the Fc fragment alone. It should be emphasized that TEM8-Fc (10 mg/kg) inhibited growth of tumors derived from LS-180 and MCF-7 cells (control versus 10 mg/kg TEM8-Fc: LS-180, means = 1.72 versus 0.16 g, difference = 1.56 g, 95% confidence interval [CI] = 0.96 to 2.16 g, $P<.001$; MCF-7, means = 1.12 versus 0.08 g, difference = 1.04 g, 95% CI = 0.77 to 1.31 g, $P<.001$) (Fig. 2, A) and was as efficient as VEGF-Trap in suppressing the growth of tumors derived from HepG2 cells (control versus 10 mg/kg TEM8-Fc, means = 1.28 versus 0.35 g, difference = 0.93 g, 95% CI = 0.60 to 1.25 g, $P<.001$; control versus 10 mg/kg VEGF-Trap, means = 1.28 versus 0.36 g, difference = 0.92 g, 95% CI = 0.69 to 1.13 g, $P<.001$.) (Fig. 2, B). In addition, we found that liver metastasis of the tumors in TEM8-Fc–treated mice was reduced. Liver metastasis of xenograft MCF-7 tumors was observed in four of seven mice in the control group but in none of the seven mice in the 10.0 mg/kg TEM8-Fc–treated group. Thus, we demonstrated for the first time, to our knowledge, that the extracellular domain of TEM8 fused to Fc portion of IgG1 has potent anticancer activities. As expected, immunohistochemical examination of CD31 showed a marked decrease of microvessel density in TEM8-Fc–treated tumors (Fig. 2, C and D). It is interesting to note that the malignant phenotype of tumors in TEM8-Fc–treated mice was changed, as evidenced by decreased expression of carcinoembryonic antigen in tumor tissues and the obvious change of tumor cell morphology (Fig. 2, E). Binding unknown components of the extracellular matrix and affecting tumor cell differentiation might be the possible mechanisms for its effect on tumor phenotype. In fact, it has been demonstrated that TEM8 interacts with the cleaved C5 domain of collagen α3(VI), an important component of extracellular matrix (21). The precise mechanisms underlying TEM8-Fc–induced phenotype change of tumor need to be further explored.

Using TEM8-Fc as bait, we searched for the natural interacting partners of TEM8 to further elucidate the molecular basis for its anticancer activity. A distinct protein band of about 55 kDa was trapped by TEM8-Fc (Fig. 3, A, dotted arrow). We then analyzed this band by trypsin digestion and mass spectrometry. Several proteins were identified whose sequences were consistent with those of the tryptic fragments obtained from this band. Further Q-TOF Ultima hybrid quadruple time-of-flight mass spectrometry assay detected one tryptic peptide, whose amino acid sequence (EAEAAIFHRQLFEELR) was 100% identical to the amino acid residues 379–385 of M2 isoenzyme of pyruvate kinase (M2-PK). Using an anti–M2-PK antibody, we found that a distinct protein band of about 55 kDa also could be pulled down from the homogenate of HepG2 tumor (Fig. 3, B). This band could be immunoblotted by TEM8-Fc (Fig. 3, C), indicating that M2-PK is an interacting partner of TEM8. M2-PK is an isoenzyme of PK that is predominantly found in tumor cells and therefore is termed as tumor M2-PK (22). Accumulated data suggest that M2-PK may play an important role in tumor growth and metastasis (23). Tumor M2-PK can directly interact with various oncoproteins and can also be released from tumor cells into peripheral blood; thus, it functions as a marker of the tumor load in cancer patients (24–26). From the results, we can hypothesize that rapid proliferation of tumor cells results in local hypoxia, which in turn leads to an increased expression of PK and the formation and extracellular release of M2-PK. The increased PK activity might enhance glycolysis and produce more ATP to support the survival and growth of tumor cells under the conditions of low oxygen supply. Another possibility is that the released M2-PK might stimulate angiogenesis by binding to TEM8 and thus improve the hypoxia status.

Taken together, our results indicate that TEM8 is an attractive target for targeted cancer therapy and TEM8-Fc would be a potent antitumor agent. However, extensive preclinical and clinical studies are necessary before TEM8-Fc can enter clinical trials.

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