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QUARTERLY

Combined delivery of an antiangiogenic protein (angiostatin) and an immunomodulatory gene (interleukin-12) in the treatment of murine cancer✪

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We investigated the feasibility of a novel therapeutic approach to treat neoplastic diseases in mice. This novel strategy consists in delivering a protein (angiostatin) with strong antiangiogenic properties, followed by administration of the interleukin 12 gene that is strongly immunomodulatory and has also some antiangiogenic effects. When angiostatin-mediated antiangiogenic therapy was used in combination with intratumor delivery of the IL-12 gene (a strategy much safer than IL-12 protein administration), this produced a synergistic therapeutic effect.

Formation of vasculature in primary tumors and metastases, i.e. tumor angiogenesis, plays an important role in the development of neoplastic diseases. Inhibition of angiogenesis in primary tumors and metastases is the basis of the antiangiogenic strategy in gene cancer therapy models.

At present, many endogenous polypeptide inhibitors of angiogenesis are known that in a highly specific manner prevent the development of blood vessels in primary tumors and metastases. Many have been used in experimental studies. One of the widely-known angiogenesis inhibitors is angiostatin discovered by Folkman and coworkers [1]. It is a proteolytic 38-kDa fragment of plasminogen, containing four plasminogen kringle domains (K1–4) [1]. Angiostatin inhibits proliferation of endothelial cells, without affecting proliferation of neoplastic cells [2]. It causes apoptosis of endothelial cells forming part of blood vessels' structure [3]. Angiostatin

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Abbreviations: BAE, bovine aortal endothelial; FCS, fetal bovine serum; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

blocks ATP synthesis in these cells by binding to ATP synthase subunits present on external membranes of endothelial cells [4]. It has been shown in various tumor models that angiostatin inhibits development of primary tumors and metastatic spread by preventing angiogenesis [1, 5–7].

An enhanced antitumor effect of concomitant angiostatin administration and radiotherapy has been observed [8, 9]. Synergistic effects have been described for angiostatin/endostatin use [10]. In therapeutic experiments, angiostatin derived from plasminogen digestion, recombinant protein, or angiostatin-encoding gene have all been used [11].

In our studies we attempted a novel strategy consisting of a combined delivery of an antiangiogenic protein together with an immunomodulatory interleukin gene (IL-12) delivered directly to the tumor site.

Interleukin 12 has strongly immunomodulatory properties such as the ability to stimulate lymphocytes T and the production of interferon γ (IFNγ), proliferation and activation of NK cells, as well as the cytotoxicity of LAK cells and TIL cells. This cytokine stimulates the production of tumor necrosis factor TNFα [12]. In addition, IL-12 shows antiangiogenic properties that result from the induction of IP-10, a protein with strong antiangiogenic properties. IL-12 is a short-lived toxic protein and administration of its gene enables lowering the levels of the circulating IL-12 protein without compromising its therapeutic effects [12–14].

The results of our experiments suggest that combined delivery of an antiangiogenic protein and an immunomodulatory gene produce an enhanced therapeutic effect (i.e. stronger inhibition of tumor growth).

MATERIALS AND METHODS

Plasmids. The pBCMGSNeo/IL-12 plasmid containing sequences of both IL-12 subunits (p35 and p40) separated by an IRES sequence and under the control of the cytomegalovirus promoter was obtained from Dr. H. Yamamoto, Osaka University (Japan) [15]. As a control plasmid we used pBCMGSNeo obtained from Dr. H. Karasuyama from the Institute of Immunology in Basel (Switzerland).

Cell lines. B16(F10) murine melanoma cell line was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FCS) (GibcoBRL), at 37°C and under 5% CO_2. Bovine aortic endothelial cells were obtained from Clonetics (San Diego, CA, U.S.A.) and cultured in RPMI + 5% FCS (Gibco-BRL).

Animals. Six-to-eight-week-old C57Bl/6 mice were from an on-site animal facility. Mice had their left dorsal side shaved and were inoculated with 2.5×10^6 B16(F10) cells in 100 μl PBS^- (without Ca^{2+} and Mg^{2+})/animal. Tumor size was measured using calipers and calculated from the formula: Volume = $(\text{Width})^2 \times \text{Length} \times 0.52$ [5].

Isolation of plasminogen. Plasminogen was isolated from human serum as described in a published procedure [5, 6]. Serum (outdated lots) was obtained from the Regional Blood Supply and Therapy Center in Katowice (Poland).

The serum was thawed and centrifuged at 7500 r.p.m. for 30 min at room temperature. Following this the supernatant was filtered using 0.45 μm Millipore filters. The serum was then resuspended in 50 mM phosphate buffer (pH 7.5) at a volume ratio of 1:2 and applied onto an L-lysine-Sepharose 4B column (Amersham Pharmacia); bed volume = 36ml. Separation was carried out at 4°C. Next, the column bed was washed with 0.5 M NaCl in order to elute non-specifically bound substances. The bound plasminogen was eluted using 0.2 M ε-amine-n-capronic acid. The plasminogen obtained was then dialyzed at 4°C against 20 mM Tris/HCl (pH 7.6) for 48 h using MWCO (Molecular Weight Cut-off) 8000 dialysis bags. Finally, the plasminogen preparation was lyophilized.

Isolation of angiostatin. Angiostatin was obtained from plasminogen by digesting it

with porcine pancreas elastase (ICN, Costa Mesa, CA, U.S.A.) [5, 6]. The lyophilized plasminogen was dissolved in 50 mM phosphate buffer (pH 7.5) at 0.5 mg/ml. An appropriate amount of the enzyme was then added (0.8 elastase units per 1 ml of the plasminogen solution) and the sample was incubated at 37°C for 5 h with vigorous shaking (225–250 r.p.m.).

Angiostatin was isolated chromatographically on a lysine-Sepharose column (see above). Fractions containing angiostatin were eluted with 0.2 M ε-amine-n-capronic acid.

The obtained angiostatin was dialyzed for 24 h against 50 mM phosphate buffer (pH 7.5) using 15000 MWCO dialysis bags. Next, it was dialyzed for 24 h against deionized water and lyophilized. Subsequently, angiostatin was identified electrophoretically as described below (on a 10% polyacrylamide gel containing SDS followed by Western blotting. Prior to use, angiostatin was suspended in PBS^- (without Ca^{2+} and Mg^{2+}) buffer.

Identification of angiostatin by polyacrylamide/SDS gel electrophoresis. Thirty-microgram aliquots of plasminogen and angiostatin were separated elecrophoretically on a 10% polyacrylamide gel containing 0.4% SDS. The resolved SDS/PAGE gel was stained with Coomassie and destained with a 45% methanol/10% acetic acid mixture. High Molecular Weight Standard Mixture, egg albumin and bovine albumin (Sigma-Aldrich) was used as size marker.

Analysis of angiostatin using Western blotting. Samples of plasminogen and angiostatin were electrophoresed in a 12% polyacrylamide gel containing SDS and transferred onto nitrocellulose membrane (Schleicher & Schuell). Protein bands were visualized with Ponceau Red staining. After destaining with Tris-buffered saline (TBS; 150 mM NaCl, 10 mM Tris/HCl, pH 7.4) containing 0.1% Tween-20, the membrane was incubated in blocking buffer (TBS/3% skim milk) for 1 h at room temperature. The membrane was incubated at 4°C overnight with monoclonal antibodies $36E_6$ raised against human plasminogen kringle 1–3 (obtained from Dr. H.R. Lijnen, Center of Molecular and Vascular Biology, Leuven, Belgium) diluted to the final concentration of 50 μg/ml with TBS/3% skim milk. On the following day the membrane was washed five times with TBS/0.1% Tween-20 and then incubated for 1 h at room temperature with biotinylated anti-mouse IgG antibodies (Vector Laboratories, Inc., Burlingame, CA, U.S.A.) diluted 1:200 with TBS/1% skim milk. After five washes in TBS the membrane was incubated for 45 min at room temperature with an avidin–biotinylated horseradish peroxidase complex (Vector Laboratories) diluted 1:100 in TBS. The peroxidase substrate used for band staining was 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich).

Endothelial cell proliferation assay. The assay was performed according to the method of Lucas *et al.* [3] with several modifications. Bovine aortal endothelial (BAE) cells were dispersed in a 0.05% trypsin solution and resuspended with RPMI containing 5% FCS. 5×10^3 cells in 1 ml were added to each well of gelatinized 24-well plates and incubated at 37°C for 2 h. The medium was replaced with 0.5 ml fresh RPMI containing 2% FCS and angiostatin samples. After 1-h incubation medium was added to obtain a final volume of 1 ml of RPMI + 2% FCS and a final bFGF concentration (R&D Systems, Inc., Minneapolis, MN, U.S.A.) of 10 ng/ml. After 72 h of incubation, the number of living cells was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test (Sigma-Aldrich) and absorbance was measured at 570 nm.

Animal studies. Angiostatin was injected twice a day for a 10-day period counting from the 7th day following animals' inoculation with cancer cells, when the tumor diameter had reached about 3 mm. Angiostatin was delivered in 100 μl aliquots in physiological saline buffered with phosphate, without Ca^{2+}

and Mg^{2+} (PBS^{-}). The amount of protein used was 1mg per animal per 12 h.

C57Bl/6 mice were inoculated with 2.5 × 10^6 B16(F10) melanoma cells/animal. Mice receiving combined therapy were injected intradermally 1 mg of angiostatin every 12 h for four days starting from the 7th day of the experiment and then, intratumorally, the pBCMGSNeo/IL-12 plasmid for eight days starting from the 11th day of the experiment. Mice receiving single agent therapy received either angiostatin only (from the 7th until the 11th day) or the therapeutic gene-containing plasmid DNA only (from the 11th until the 18th day). Mice from the control groups received either a plasmid without the therapeutic gene, or PBS^{-}. Aliquots of 1 μg protein were injected in 100 μl PBS^{-} every 12 h. Aliquots of 50 mg of plasmid DNA were administered intratumorally in 100 μl Ringer's solution with 0.05% lactose.

RESULTS AND DISCUSSION

Purification and identification of angiostatin obtained from human plasminogen

Angiostatin obtained from human plasminogen by enzymatic digestion with porcine pancreas elastase was chromatographically isolated on an L-lysine-Sepharose column and identified on the basis of its electrophoretic mobility in a polyacrylamide gel.

Bands of interest, i.e. those of angiostatin with the molecular mass of 38 kDa and of plasminogen with the molecular mass of 90 kDa, were identified by Coomassie staining (Fig. 1A).

Angiostatin was also identified using Western blotting. Figure 1B shows the identification of a protein corresponding to angiostatin with the molecular mass of 38 kDa.

The angiostatin obtained had antiangiogenic activity *in vitro* as shown in the endothelial cells proliferation assay (Fig. 2).

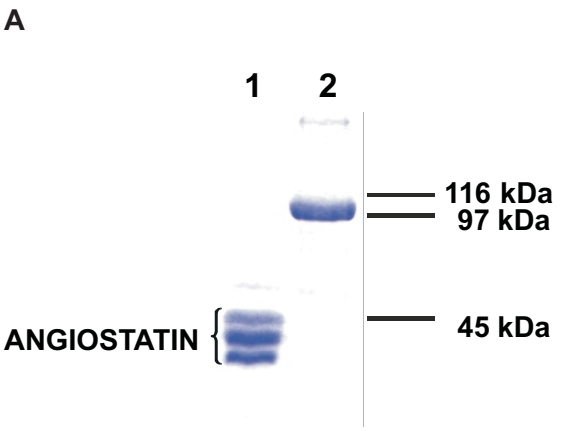

Figure 1A. Electrophoretic separation of angiostatin and plasminogen.

Electrophoresis was carried out in 10% polyacrylamide gel with SDS. Following separation, the gel was stained with Coomassie Brillant Blue. Aliquots of 30 μg of angiostatin (1) or plasminogen (2) were separated.

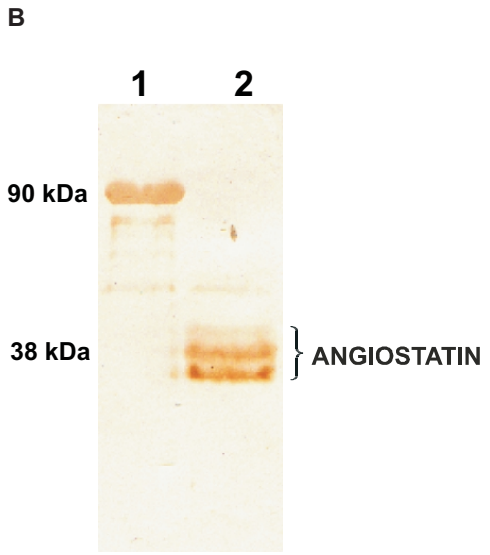

Figure 1B. Identification of angiostatin using Western blotting.

Following electrophoretic separation angiostatin was identified using a specific antibody (Western blot). To identify angiostatin, mouse monoclonal antibodies ($36H_6$) were used. Lanes: 1, plasminogen; 2, angiostatin.

In order to confirm the antiangiogenic properties of the isolated protein a therapeutic *in vivo* experiment was carried out. Syngeneic C57Bl/6 mice previously inoculated with B16(F10) cells were treated by intradermal

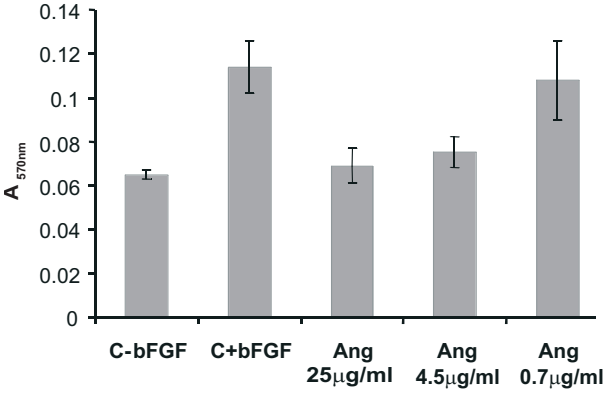

Figure 2. Inhibition of bFGF-induced proliferation of bovine endothelial (BAE) cells.

Human plasminogen-derived angiostatin (Ang) was added to BAE cells at concentrations indicated. C-bFGF, control without bFGF; C+bFGF, control with bFGF added to the final concentration indicated. Cell proliferation was quantified by the MTT assay. Results are shown as means of quadruplicate wells (±S.D.).

near-tumor-site administration of angiostatin. As a result of therapy, a significant decrease of the tumor growth rate was observed in the group of mice obtaining angiostatin, compared to the control group receiving PBS^- only (Fig. 3). When angiostatin administration was ceased, a rapid resumption of tumor growth was observed in the treatment group, so that after several days tumor size differences between the experimental and control groups became statistically insignificant (data not shown).

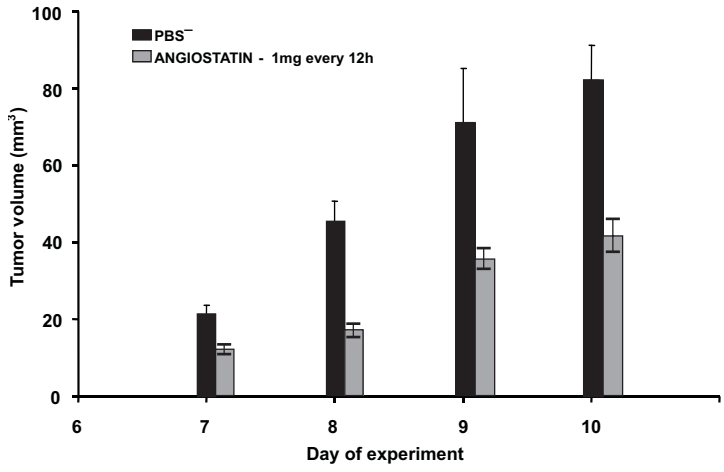

Figure 3. Inhibition of tumor growth in C57Bl/6 mice inoculated with B16-(F10) melanoma cells, following intradermal administration of angiostatin.

C57Bl/6 mice were inoculated with 2.5×10^6 B16(F10) melanoma cells/animal. Mice from the treatment group received intradermally 1 mg of angiostatin every 12 h for four days starting from the 7th day of the experiment. Control mice received PBS^- only. Each group numbered 4 animals. Each data point represents mean tumor volume (±S.D.) during consecutive days of the experiment.

Combined therapy with angiostatin and interleukin-12 gene

C57Bl/6 mice inoculated with B16(F10) murine melanoma cells were treated by intradermal administration of angiostatin followed by intratumoral administration of plasmid DNA containing the murine interleukin 12 (IL-12) gene.

The protein was administered intradermally near the tumor site using 1 mg of protein per animal per 12 h, beginning from the 7th day following the animal's inoculation with cancer cells. Injections were continued on the 8th, 9th and 10th day of the experiment.

Gene therapy consisting in daily intratumor administrations of the pBCMGSNeo/IL-12 plasmid containing the murine interleukin 12 gene was started on the 11th day of the experiment and continued until the 18th day. DNA was injected in 50 μg aliquots using 100 μl of Ringer's solution with 0.05% lactose added. A clear therapeutic effect (tumor growth arrest) was seen in the animal groups receiving angiostatin followed by IL-12 gene delivery, as compared to the control groups receiving only protein or only plasmid DNA (Fig. 4). Mice from negative control group received PBS^- or Ringer's solution with lactose (the latter not shown).

The results of our study confirm the therapeutic properties of angiostatin obtained by enzymatic digestion of human plasminogen. Inhibition of tumor growth can be observed

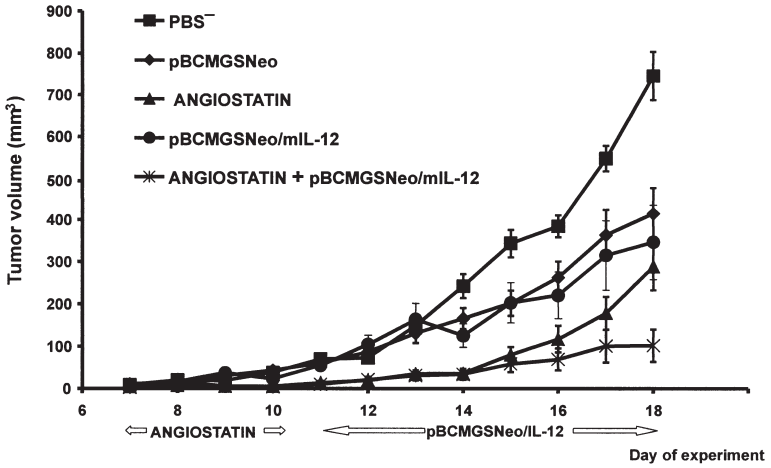

Figure 4. Inhibition of tumor growth in C57Bl/6 mice inoculated with B16(F10) melanoma cells, following combined protein therapy with angiostatin and gene therapy with interleukin 12.

C57Bl/6 mice were inoculated with 2.5×10^6 B16(F10) melanoma cells/animal. Mice receiving combined therapy were injected intradermally angiostatin every 12 h for four days starting from the 7th day of the experiment and then, intratumorally, the pBCMGSNeo/ IL-12 plasmid for eight days starting from the 11th day of the experiment (✳). Mice receiving single agent therapy were injected either angiostatin only (▲) or the therapeutic gene-containing plasmid DNA only (●). Mice from the control groups received either a plasmid without the therapeutic gene (◆), or PBS^- (■). The experimental groups numbered 5 animals each. Each data point represents mean tumor volume (±S.D.) during consecutive days of the experiment.

following treatment of affected mice with the strongly antiangiogenic angiostatin.

The antitumor effect of angiostatin stems from the inhibition of formation of blood vessels. These are necessary for growth of primary tumors and subsequent metastases.

Despite the long half-life of angiostatin in circulation (in mice up to 2.5 days) [1], the best therapeutic effect is obtained when the protein is readministered every 12 h. To obtain a distinct therapeutic effect it is necessary to apply large doses of angiostatin, about 2 mg per 24-h period. Smaller doses of the protein did not yield therapeutic results (data not shown).

The therapeutic effect obtained in treating cancer-affected mice with angiostatin only is temporary. Following cessation of therapy, rapid tumor growth is resumed. Since human angiostatin inhibits proliferation of endothelial cells in a dose-dependent manner, this most likely reduces the number of tumor cells but does not destroy them *in toto*.

Since antiangiogenic preparations are cytostatic by nature, it seemed worthwhile to investigate the outcome of a strategy combining antiangiogenic as well as some other treatment. We thus investigated the therapeutic effectiveness of delivering angiostatin with concomitant delivery of the immunomodulatory interleukin 12 gene.

Extensive studies of interleukin 12 antitumoral effects have shown that they rely upon immunomodulatory properties, especially on the stimulation of T lymphocytes as well as formation of IFNγ [12].

Our results confirm the efficacy of the adopted approach. Mice from the group receiving both angiostatin and IL-12 clearly showed a therapeutic effect, manifested by tumor growth arrest. Mice receiving only the protein or only the DNA experienced a much smaller therapeutic effect (Fig. 4). Similar results were noted previously for adenoviral-mediated concurrent administration of IL-12 and angiostatin genes (54% of total cures), compared to the administration of the IL-12 gene only (13% of complete cures) [16].

The antitumoral effects of angiostatin and interleukin 12 take place on different levels.

Angiostatin targets normal endothelial cells of blood vessels, while IL-12 acts indirectly, by activating immune system cells which in turn leads to the destruction of cancer cells. Angiostatin inhibits proliferation of endothelial cells in tumor vasculature, thus inhibiting the development of primary tumors and metastases. IL-12 mobilizes the immune system to specifically destroy cancer cells by activating and stimulating NK cells as well as lymphocytes T.

We previously used gene therapy in mice, *via* IL-12 gene transfer using naked plasmid DNA, with good result [13]. To the best of our knowledge, a concomitant use of an anti-angiogenic protein (angiostatin) and an immunomodulatory gene (interleukin 12), has never been used before to treat cancer in mice.

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REFERENCES

1. O'Reilly, M.S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R.A., Moses, M., Lane, W.S., Cao, Y., Sage, E.H. & Folkman, J. (1994) Angiostatin: A novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* **79,** 315–328.

2. Ji, W.R., Castellino, F.J., Chang, Y., Deford, M.E., Gray, H., Villarreal, X., Kondri, M.E., Marti, D.N., Llinás, M., Schaller, J., Kramer, R.A. & Trail, P.A. (1998) Characterization of kringle domains of angiostatin as antagonists of endothelial cell migration, an important process in angiogenesis. *FASEB J.* **12,** 1731–1738.

3. Lucas, R., Holmgren, L., Garcia, I., Jimenez, B., Mandriota, S.J., Borlat, F., Sim, B.K.L., Wu, Z., Grau, G.E., Shing, Y., Soff, G.A., Bouck, N. & Pepper, M.S. (1998) Multiple forms of angiostatin induce apoptosis in endothelial cells. *Blood* **92,** 4730–4741.

4. Moser, T.L., Stack, M.S., Asplin, I., Enghild, J.J., Hojrup, P., Everitt, L., Hubchak, S., Schnaper, H.W. & Pizzo, S.V. (1999) Angiostatin binds ATP synthase on the surface of human endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 2811–2816.

5. O'Reilly, M.S., Holmgren, L., Chen, C. & Folkman, J. (1996) Angiostatin induces and sustains dormancy of human primary tumors in mice. *Nat. Med.* **2,** 689–692.

6. Kirsch, M., Strasser, J., Allende, R., Bello, L., Zhang, J. & Black, P.ML. (1998) Angiostatin suppresses malignant glioma growth *in vivo*. *Cancer Res.* **58,** 4654–4659.

7. Sim, B.K.L., O'Reilly, M.S., Liang, H., Fortier, A.H., He, W., Madsen, J.W., Lapcevich, R. & Nacy, C.A. (1997) A recombinant human angiostatin protein inhibits experimental primary and metastatic cancer. *Cancer Res.* **57,** 1329–1334.

8. Mauceri, H.J., Hanna, N.N., Beckett, M.A., Gorski, D.H., Staba, M.-J., Stellato, K.A., Bigelow, K., Heimann, R., Gately, S., Dhanabal, M., Soff, G.A., Sukhatme, V.P., Kufe, D.W. & Weichselbaum, R.R. (1998) Combined effects of angiostatin and ionizing radiation in antitumour therapy. *Nature* **394,** 287–291.

9. Griscelli, F., Li, H., Cheong, C., Opolon, P., Bennaceur-Griscelli, A., Vassal, G., Soria, J., Soria, C., Lu, H., Perricaudet, M. & Yeh, P. (2000) Combined effects of radiotherapy and angiostatin gene therapy in glioma tumor model. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 6698–6703.

10. Yokoyama, Y., Dhanabal, M., Griffioen, A.W., Sukhatme, V.P. & Ramakrishnan, S. (2000) Synergy between angiostatin and endostatin: Inhibition of ovarian cancer growth. *Cancer Res.* **60,** 2190–2196.

11. Feldman, A.L. & Libutti, S.K. (2000) Progress in antiangiogenic gene therapy of cancer. *Cancer* **89,** 1181–1194.

12. Tahara, H. & Lotze, M.T. (1995) Antitumor effects of interleukin-12 (IL-12): Applications for the immunotherapy and gene therapy of cancer. *Gene Ther.* **2,** 96–106.

13. Budryk, M., Wilczyńska, U., Szary, J. & Szala, S. (2000) Direct transfer of IL-12 gene into growing Renca tumors. *Acta Biochim. Polon.* **47,** 385–391.

14. Tan, J., Newton, C.A., Djeu, J.Y., Gutsch, D.E., Chang, A.E., Yang, N.S., Klein, T.W. & Hua, Y. (1996) Injection of complementary DNA encoding interleukin-12 inhibits tumor establishment at a distant site in a murine renal carcinoma model. *Cancer Res.* **56,** 3399–3403.

15. Obana, S., Miyazawa, H., Hara, E., Tamura, T., Nariuchi, H., Takata, M., Fujimoto, S. & Yamamoto, H. (1995) Induction of anti-tumor immunity by mouse tumor cells transfected with mouse interleukin-12 gene. *Jpn. J. Med. Sci. Biol.* **48,** 221–236.

16. Gyorffy, S., Palmer, K., Podor, T.J., Hitt, M. & Gauldie, J. (2001) Combined treatment of a murine breast cancer model with type 5 adenovirus vectors expressing murine angiostatin and IL-12: A role for combined anti-angiogenesis and immunotherapy. *J. Immunol.* **166,** 6212–6217.