Enhanced Antiangiogenic Therapy of Squamous Cell Carcinoma by Combined Endostatin and Epidermal Growth Factor Receptor-Antisense Therapy

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ABSTRACT

Purpose: We tested the combined effects of antiangiogenic endostatin and epidermal growth factor receptor (EGFR) antisense gene therapy on squamous cell carcinoma (SCC).

Experimental Design and Results: The 1483 cell line of human head and neck SCC (HNSCC) and SCC-VII/SF murine SCC cells was used to establish tumors in nude mice and immunocompetent C3H mice, respectively. Tumor-bearing mice were treated with endostatin (20 mg/kg/day, s.c.), liposomal EGFR-antisense expression plasmid (25 μg/mouse, three times/week, intratumoral), a combination of both agents, or liposomal EGFR-sense plasmid as a control. Endostatin or EGFR-antisense alone significantly, yet partially, inhibited the growth of 1483 and SCC-VII/SF tumors, and a combination of both treatments completely blocked tumor growth. Immunohistochemistry analysis demonstrated that a complete suppression of tumor angiogenesis was achieved by the combination treatment. Down-regulation of vascular endothelial growth factor was shown in EGFR-antisense-treated tumors. These results suggest that the EGFR-antisense treatment, in addition to its inhibitory activity on tumor cell proliferation, might have a synergistic effect with endostatin on SCC-induced angiogenesis. In vitro studies demonstrated that EGFR inhibition by antisense oligonucleotides or EGFR-specific tyrosine kinase inhibitor down-regulated the production of VEGF in HNSCC cells. Additional experiments demonstrated that these EGFR inhibition approaches also directly suppressed the growth of endothelial cells.

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Conclusion: A combination of endostatin and EGFR targeting strategies profoundly inhibited the angiogenesis and growth of SCC in vivo. EGFR-antisense therapy might have multiple inhibitory effects against both tumor cells and endothelial cells, leading to enhanced antitumor efficacy. Such a combination strategy might represent a novel and promising approach for HNSCC therapy.

INTRODUCTION

Tumor growth and metastasis depend on blood supply and vessel formation (1). It is believed that tumor cells acquire the ability to stimulate angiogenesis, a process by which new vessels are formed from preexisting host vasculature. Angiogenic factors produced by tumor cells, including VEGF, fibroblast growth factors, and angiopeptins, promote tumor angiogenesis (1). More recently, it has been found that tumor cells also produce angiogenesis inhibitors. The angiogenic phenotype of a solid tumor is established as a result of the net balance between the activities of angiogenesis promoters and angiogenesis inhibitors. Among the identified angiogenesis inhibitors, endostatin was isolated from hemangioendothelioma cells as a COOH-terminal segment of collagen XVIII (2). Other tumor-derived endogenous antiangiogenic factors include a cleaved form of antithrombin and angiotatin, a proteolytic fragment of plasminogen (3, 4). Preclinical testing of these antiangiogenic factors in animal models suggested that they could effectively suppress the growth and/or metastasis of experimental tumors (2–4). The identification of tumor-derived antiangiogenesis inhibitors and the demonstration of their angiostatic functions have laid a rational foundation for the development of tumor-specific antiangiogenic therapy.

Achieving highly efficient angiostatic and antitumor effects with currently available antiangiogenic factors remains challenging. Although partial inhibition of tumor growth is usually seen when an antiangiogenic factor is used to treat experimental tumors, tumor regression or complete inhibition of tumor growth is rarely reproducibly achieved. Although a certain clinical benefit was shown in advanced-stage cancer patients in recent endostatin and angiostatin trials, significant antitumor efficacy has yet to be demonstrated with these antiangiogenic therapies (5). The overall antitumor effect of an angiostatic drug could be related to many factors, including the angiogenic status of a given tumor, the heterogeneity of the...
endothelium, the biochemical activity and potency of the drug used, and the routes of administration or scheduling of drug delivery. Furthermore, because tumor cells are able to generate a large quantity of potent angiogenic factors, attacking only the endothelial compartment within a tumor with one angiostatic drug as monotherapy might be insufficient to reverse tumor angiogenesis. On the basis of these considerations, we postulated that the combination of a direct antiangiogenic therapy and an approach directed against the tumor cell compartment might achieve synergistic antitumor effects.

EGFR is a transmembrane receptor that has been identified as a key regulator of cell growth. Binding of ligand to EGFR activates a series of signaling pathways that transduce the proliferating signal from the cell membrane to the nucleus (6). In addition, EGFR-mediated signaling is also associated with other malignant phenotypes, such as tumor cell invasion, metastasis, and up-regulation of angiogenic factors (6–9). EGFR overexpression is believed to play a key role in the development of solid tumors, such as HNSCC and lung cancer (10). Down-modulation of EGFR or its activity has been shown to be effective in inhibiting tumor cell proliferation as well as the production of angiogenic factors, thereby suppressing tumor growth (11–13). In the present study, we combined endostatin and EGFR-antisense gene therapy in HNSCC models and found that such a combination treatment could completely inhibit the growth of HNSCC in vivo. We also demonstrated that this combination strategy resulted in a robust blockade of tumor angiogenesis. To investigate the mechanism of this enhanced antiangiogenic effect, we conducted a series of in vitro studies and found that EGFR targeting approaches inhibited both the growth of endothelial cells and VEGF production by tumor cells.

MATERIALS AND METHODS

Cells and Plasmids. The 1483 line is a HNSCC cell line derived from a tumor of the retromolar trigone region of the oropharynx (14). Cells were cultured in DMEM supplemented with 10% FCS and antibiotics (Invitrogen/Life Technology, Gaithersburg, MD). SCC-VII/SF is a spontaneously arising SCC of C3H mice (15). The SCC-VII/SF line was maintained in RPMI 1640 containing 10% FCS and antibiotics (Invitrogen/Life Technology, Gaithersburg, MD). HUVECs were purchased from American Type Culture Collection and cultured in modified F12K medium supplemented with 0.1 mg/ml heparin, 0.03 mg/ml endothelial cell growth supplement, and 10% FCS.

Plasmids expressing the 40-bp antisense (pHU6-EAS) or sense (pHU6-ES) RNA of the human EGFR gene (nucleotides −20 to +20 corresponding to the ATG site) under the U6 promoter were constructed as described (13). The plasmid expressing murine endostatin with a histidine tag (pTBO1#4) was a kind gift from Dr. Judah Folkman (Harvard Medical School, Boston, MA) (2).

Recombinant Endostatin. Recombinant murine endostatin was expressed and purified according to a protocol previously established in the laboratory (16). Briefly, the BL21(DE3)pLysS strain of Escherichia coli transformed with plasmid pTBO1#4 were cultured in 1 liter of Luria broth, and expression of endostatin was induced with 0.3 mM isopropyl-1-thio-β-p-galactopyranoside for 3 h. Subsequently, inclusion bodies were collected by resuspending the pelleted bacteria with 100 ml of lysis buffer [0.1 M Tris-HCl (pH 8.0), 5 mM EDTA, and 50 μg/ml lysozyme], sonication in presence of 0.1% sodium deoxycholate for 15 min, and centrifuged at 8000 × g for 10 min. The collected inclusion bodies were then washed three times with 100 ml of lysis buffer plus 0.1% sodium deoxycholate, followed by centrifugation at 8000 × g for 10 min. The inclusion bodies were dissolved in 30 ml of 0.05 M Tris (pH 8.0), 1% sodium N-laurylsarcosine, and 1 mM DTT and centrifuged at 8000 × g for 10 min at 4°C. The obtained clear supernatant was then dialyzed at 4°C twice against 1500 ml of 0.05 M Tris-HCl (pH 8.0) and 0.1 mM DTT for 4 h, twice against 1500 ml of 0.05 M Tris-HCl (pH 8.0), once against 1000 ml of 0.05 M Tris-HCl (pH 8.0), 0.01 mM oxidized glutathione, and 1 mM reduced glutathione for a 4-h cycle, and finally, once against 0.05 M Tris-HCl (pH 8.0) for 5 h. The dialysis product was subject to endotoxin level determination. The purified protein was finally quantitated by the Bio-Rad protein dye method as described by the manufacturer, aliquoted, and stored at −20°C.

In Vivo Tumor Studies. For the syngeneic tumor model and the HNSCC xenograft model, 1 × 10^6 SCC-VII/SF cells or 1483 cells were inoculated s.c. at a dorsal site on 4–6-week-old C3H/HeJ mice or nude mice nu/nu (Jackson Laboratory, Bar Harbor, ME), respectively. When the tumor nodules reached 5 mm in diameter (approximately 10–14 days after inoculation), mice were randomly assigned to treatment groups (PBS alone, endostatin alone, pHU6-EAS (EGFR-antisense construct) complexed with liposome, endostatin plus pHU6-EAS complexed with liposome, and pHU6-EAS (EGFR-sense construct) complexed with liposome). There were five mice in each treatment group. Endostatin was injected s.c. at a site distal to the tumor inoculation site (20 or 40 mg/kg/day). Treatment with the EGFR-antisense U6 construct or the EGFR-sense U6 construct was performed by mixing the plasmid DNA (25 μg) with DC-Chol liposomes (50 nmol) in a volume of 50 μl for each mouse and injecting intratumorally three times/week. Tumors were measured with calipers, and tumor volumes were calculated (tumor volume = length × width^2 × 0.52). The mice were sacrificed when tumors reached 2.0 cm in diameter or became ulcerated, as per the protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee, and the tumors were resected. The inhibition rate of endostatin (20 mg/kg/day) at the experiment end point (14th day) was calculated as:

Inhibition rate = Mean of control tumor volumes –
\[\text{mean of endostatin-treated tumor volumes}\] × 100
\[\text{Mean of control tumor volumes}\]

Immunohistochemistry Analysis for Microvessel Formation. Tumor specimens were fixed and frozen in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC). Five-μm cryosections were cut and stained with H&E for histopathological analysis. To analyze the microvessel formation in tumors, sections were stained with anti-CD31 monoclonal antibody (DAKO Corp., Carpinteria, CA) and subsequently with the ABC method. Positively stained vascular endothelial cells (brown) were visualized and imaged using a digital camera attached to an Olympus microscope. The micro-
vessel density was determined according to methods described previously (17). Briefly, regions of highest vessel density (“hot spot” regions) were scanned at low magnification (×40–100) and counted at higher magnification (×200). Three such “hot spot” fields were counted in each tumor section, and the mean microvessel density value was recorded. Any endothelial cell or endothelial cell cluster that was clearly separated from adjacent microvessels was considered a single, countable microvessel. Positively stained vascular endothelial cells were visualized and imaged using a Magnifire camera (Olympus, Melville, NY) attached to an Olympus Provis microscope.

**Western Blot Analysis.** At the experimental end point, tumor nodules were resected and homogenized. Briefly, frozen tumor tissue samples (0.3–0.5 g) were processed in ice-cold PBS, followed by high speed homogenization in 1.5 ml of lysis buffer (20 mm Tris, 150 mm NaCl, 1% NP40, 10% glycerol, 5 μg/ml leupeptin, 10 μg/ml aprogin, and 0.2 mm phenylmethylsulfonyl fluoride) until no tissue chunks remained (1–2 min). Homogenized samples were then set on ice for 15–20 min with constant shaking and subsequently centrifuged at 10,000 g for 20 min at 4°C. Equal amounts of protein were assayed for protein concentration with the Bio-Rad protein dye before being subjected to SDS-PAGE separation on an 8% PAGE gel under reducing conditions, according to a standard method (13). Immunoblotting was performed using mouse antihuman EGFR monoclonal antibody (BD Transduction Laboratory), detected using peroxidase conjugated goat antimouse IgG and chemiluminescence detection kit (Pierce), and visualized using Kodak BiomaxMR film (13). The intensities of the signals were normalized by reprobing the stripped membrane with anti-β-actin antibody.

**Transfection Experiments.** Transfection with EGFR antisense or sense oligonucleotides was performed using LFA liposome (Invitrogen) and the protocol provided by the manufacturer. The phosphorothioate oligonucleotides used in the transfection experiments were commercially synthesized according to sequences published previously (18, 19). Two EGFR antisense oligonucleotides (AS02 and AS03) and two EGFR sense oligonucleotides (sense 02 and sense 03) with the following sequences were used: CCCCCAGCTCCATTTGGG (AS02), CCTCCGTGTCTGCTCC (AS03), CCATTGGGAGCTGCTGGGG (SENSE02), and GGAGCATGACCACGAG (sense 03). AS02 and AS03 were tested and confirmed to effectively inhibit EGFR expression in cell culture when transfected (18, 19). One day before transfection, 1483 HNSCC cells and HUVECs were transferred to 24-well plates at a density of 8 × 10^4 cells/well. For transfection, the culture medium was replaced with 0.5 ml of serum-free OPTI-MEM transfection medium (Invitrogen), followed by the addition of 1 μg of oligonucleotide mixed with 1 μl of LFA. As a control, LFA alone was used to treat cells. After a 6-h incubation at 37°C, the transfection medium was replaced with culture medium with 10% FCS.

**Treatment of Cultured Cells with EGFR-specific TKI PD153035.** 1483 cells or HUVECs were inoculated in 24-well plates at a density of 8 × 10^4 cells/well. EGFR-TKI PD153035 was purchased from CalBiochem and dissolved in DMSO (20). For the treatment, PD153035 (or DMSO) was added to the culture at a dose shown previously to inhibit EGFR tyrosine phosphorylation in HNSCC cells (21). The cells were incubated at 37°C until subsequent assays.

**Effect of EGFR Antisense Oligonucleotides or PD153035 on HUVEC Growth.** HUVECs transfected with EGFR antisense/sense oligonucleotides or LFA alone were incubated at 37°C for 48 h after the posttransfection medium change. HUVECs treated with PD153035 or DMSO were incubated at 37°C for 48 h in presence of the drug. Subsequently, cells were trypsinized from the 24-well plates, stained with the trypan blue dye, and counted via vital dye exclusion. Each data point was presented as the mean ± SD of four replicate wells.

**Detection of VEGF in Cell Culture and Tumor Homogenates.** Six, 12, and 48 h after the posttransfection medium change, supernatants were taken from the 1483 cells transfected with EGFR antisense/sense oligonucleotides or LFA alone. For the PD153035-treated 1483 cells, supernatants were taken after 12, 18, and 48 h of treatment. VEGF concentrations in the supernatants were determined by ELISA using a kit from R&D system. The ELISA assays were performed according to the manufacturer’s manual. Each data point was presented as the mean of triplicate wells. To quantitate VEGF levels in tumor homogenates, lysates obtained from the homogenized tumor tissues were subjected to the ELISA assay.

**Statistical Analysis.** For in vivo experiments, tumor volumes were presented as mean ± SE. The Student’s t test was used to examine the statistical significance of the differences between groups (two-tailed). The level of significance will be set at P < 0.05.

**RESULTS**

**The Antiangiogenic and Antitumor Effects of Endostatin on SCC Tumors.** To test whether endostatin inhibits the growth of HNSCC, both an SCC-VII/SF syngeneic model and a human HNSCC xenograft model (1483) were used in this study. When the SCC-VII/SF tumor-bearing C3H mice were treated with recombinant endostatin (s.c., 20 mg/kg/day), the growth of SCC tumors were significantly inhibited by 46% at the experimental end point as compared with the PBS control (Fig. 1A). Similar inhibitory effects were observed in the 1483 HNSCC xenograft model, with an inhibitory rate of 53% at the experimental end point (Fig. 1B). As shown in Fig. 1, increasing the endostatin dose from 20 to 40 mg/kg/day did not increase the overall antitumor effect in either model. Although endostatin has been shown to be inhibitory to the growth of a variety of experimental tumors, it had not been studied previously in HNSCC. Thus, our results represent the first demonstration that antiangiogenic endostatin suppressed the growth of HNSCC tumors.

**Combined Effect of Endostatin and EGFR-Antisense Gene Therapy on 1483 HNSCC Xenografts.** As shown in above experiment, moderate suppression of HNSCC growth by endostatin treatment alone was observed in this study. To determine whether EGFR down-modulation could enhance the anti-HNSCC effect of endostatin, we tested the combined effect of an EGFR-antisense construct and antiangiogenic endostatin on the growth of established HNSCC tumors. It was demonstrated previously that liposome-mediated EGFR antisense therapy inhibited the growth of xenografted HNSCC tumors more...
Fig. 1 Effect of endostatin on the growth of SCC-VII/SF and 1483-HNSCC tumors. For the SCC-VII/SF tumor model (A), 1 x 10^6 SCC-VII/SF cells were inoculated s.c. into the flank of each C3H mouse. To establish the 1483 human HNSC xenograft, 1 x 10^6 1483 cells were implanted in the same manner. When tumors grew to 5 mm in diameter (usually 10–14 days after tumor cell implantation), PBS, endostatin (20 or 40 mg/kg/day) was injected s.c. at a distal site, respectively. Five mice were used per group. Mice were sacrificed when tumors reached 2.0 cm in diameter or became ulcerated. Tumors were measured as Length x Width^2 x 0.52 and presented as means; bars, SE. The last two time points (13th and 14th days), the differences between endostatin (20 mg/kg, daily) and PBS control are significant (P < 0.01), and so are the differences between high-dose endostatin (40 mg/kg, daily) and PBS control at these two time points (P < 0.01). There is no significant difference between low- and high-dose endostatin treatment at any time point. Each experiment was performed twice with similar results.

Fig. 2 Combined effect of endostatin and EGFR-antisense on SCC-VII/SF and 1483-HNSC tumors. One million SCC-VII/SF cells (A) or 1483 cells (B) were inoculated into C3H or nude mice, respectively. Treatments were started when tumors reached 5 mm in diameter. Endostatin (20 mg/kg, daily; Endo) was injected s.c. at a site distal to the implanted tumor. EGFR-antisense plasmid DNA (AS) mixed with Lipo-fectamine was injected intratumorally at 25 μg/mouse, three times/week. For the control group, EGFR-sense plasmid DNA (Sense; Ref. 13) was injected intratumorally at 25 μg/mouse, three times/week. Five mice were used for each group in each experiment. Tumors were measured as Length x Width^2 x 0.52 and presented as means; bars, SE. Each experiment was performed twice with similar results.

Efficiently than naked plasmid DNA, and that 25 and 50 μg of EGFR-antisense plasmid DNA were equally effective (13). In the current experiment, we compared the tumor-inhibitory effects of endostatin alone (20 mg/kg/day), EGFR-antisense construct alone (25 μg), and the combination of both agents. As shown in Fig. 2A, a combination of endostatin and EGFR-antisense therapy achieved nearly complete inhibition of 1483 HNSC xenograft tumors, whereas each monotherapy alone resulted in moderate suppression of tumor growth as compared with the vehicle-treated tumors. Liposomal EGFR-sense treatment did not alter the growing rate of the 1483 xenografted tumors, indicating that the EGFR-antisense therapy was specifically directed against EGFR. To determine whether the anti-tumor effect of endostatin and EGFR-antisense therapy was associated with suppression of angiogenesis, the status of vessel formation in treated 1483 xenografts was assessed by immunohistochemistry with endothelial cell-specific CD31 (PE-CAM) staining. A representative result of immunostaining is shown in Fig. 3. It was found that a high density of microvessels was present in untreated 1483 HNSC tumors, whereas endostatin or EGFR-antisense treatment alone resulted in significant reduction in vascularization (Fig. 3, A–F; Table 1). When endostatin and EGFR-antisense were used together, a complete suppression of tumor angiogenesis was achieved (Fig. 3, G and H; Table 1).

Combined Effect of Endostatin and EGFR-Antisense Gene Therapy on SCC-SV/SF Tumor Growth in Syngeneic C3H Mice. We further tested the combined effects of endostatin and EGFR-antisense on the SCC-VII/SF syngeneic model. In this experiment, tumor-bearing C3H/HeJ mice were treated with the same protocols as used in the 1483 xenograft model. The results presented in Fig. 2B show that the combination of endostatin and EGFR-antisense treatment almost completely blocked tumor growth, whereas each monotherapy only led to partial inhibition when applied alone. Thus, use of an independent immunocompetent animal model verified the synergistic antitumor activity of endostatin and EGFR-antisense therapy.

Effect of EGFR Antisense Oligonucleotides or PD153035 on the Production of VEGF by 1483 HNSC Cells. In vivo experiments showed that EGFR-antisense therapy significantly reduced the vessel density in 1483 HNSC xenografts and SCC-VII/SF SCC tumors. VEGF is a major proangiogenic factor in tumor angiogenesis. EGFR activation has been shown to up-regulate VEGF expression in tumor cells (9). In this study, we tested the effect of EGFR down-modulation on VEGF expression in cultured 1483 cells using two independent approaches, i.e., EGFR-antisense
oligonucleotides and the EGFR-selective TKI PA153035. As shown in Fig. 4, either of two EGFR-antisense oligonucleotides (AS02 and AS03) modestly inhibited VEGF production by 1483 HNSCC cells at 18 h after transfection and markedly inhibited the VEGF production by 48 h after transfection as compared with the EGFR-sense oligonucleotides or liposome vector control. Similarly, 0.1 and 1 μM PD153035 significantly inhibited the ability of 1483 cells to produce VEGF.

Fig. 3 Immunohistochemistry analysis of treated tumors. Representative views of anti-CD31 antibody stained vascular endothelium (brown). A and B, EGFR-sense; C and D, endostatin alone; E and F, EGFR-antisense alone; G and H, endostatin plus EGFR-antisense. The left panel (A, C, E, and G) is the lower power view (×100; scale bar, 100 μm), and the right panel shows the higher power view (×400; scale bar, 25 μm).
These results suggest that EGFR signaling might contribute to the angiogenic phenotype of 1483 HNSCC cells, and that EGFR inhibition down-regulates the angiogenic activity of 1483 cells.

**Down-Modulation of EGFR and VEGF by EGFR-Antisense Treatment in Vivo.** To test whether the EGFR-antisense gene therapy effectively down-modulated the expression of EGFR in vivo, we collected the tumor nodules from the treated animal and examined the level of EGFR in the lysates of the homogenized tumors using Western blot analysis. Fig. 5 demonstrated that EGFR antisense treatment alone or in combination with endostatin significantly reduced the expression of EGFR in 1483 HNSCC tumors. This result confirmed that EGFR-antisense gene therapy could be an effective approach to down-modulate EGFR in vivo, as shown in previous studies (13).

As shown in our in vitro studies, inhibition of EGFR expression or activation significantly down-regulated VEGF in 1483 HNSCC cells. To further determine whether such antiangiogenic effect also occurred in vivo, we examined the levels of VEGF in the tumor homogenates. As shown in Fig. 5, the levels of VEGF in tumors treated with EGFR-antisense, alone or combined with endostatin, were significantly reduced. In contrast, neither EGFR-sense control nor endostatin monotherapy resulted in alteration of VEGF levels. These results suggest that EGFR signaling might be important for the maintenance of angiogenic stimulation in HNSCC tumors, and that EGFR targeting could be an effective approach to reduce the “angiogenic load” in HNSCC.

**Effect of EGFR-Antisense Oligonucleotides or PD153035 on HUVEC Growth.** The direct effect of EGFR down-modulation on endothelial cell growth was examined using EGFR-antisense oligonucleotide AS02 as well as EGFR-TKI PD153035. Results presented in Fig. 6 demonstrate that 0.5 or 1 µg of AS02 significantly inhibited the growth of HUVEC. In contrast, Lipofectamine alone or EGFR-sense oligonucleotide did not modulate HUVEC growth. In the EGFR-TKI experiment, 50 nM PD153035 largely suppressed HUVEC growth in culture, suggesting that EGFR signaling might directly contribute to endothelial cell growth.

**Table 1** MVD in excised tumors

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<th>Treatment</th>
<th>MVDa</th>
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<tr>
<td>EGFR-sense</td>
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<tr>
<td>EGFR-antisense</td>
<td>35.33 ± 3.34</td>
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<tr>
<td>Endostatin</td>
<td>19.33 ± 2.05</td>
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<td>Combination</td>
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a 1483 HNSCC tumors were resected from mice at the experimental end point. Resected tumors were sectioned and stained as described in the text. Microvessel densities (MVD) were determined by counting CD31-positive endothelial cells in the sections and presented as mean ± SE positive cells/field determined from measurements of three “hot-spot” fields (17).

b P of each treatment was calculated using Student’s t test (two-tailed).

**DISCUSSION**

The results of this study suggest that the antiangiogenic protein endostatin significantly inhibits the growth of established HNSCC tumors, and that the antitumor effects of endostatin synergize with the EGFR-down-modulation therapy, leading to substantial suppression of HNSCC growth.

Endostatin has been shown to significantly inhibit tumor vascularization and the growth of a wide variety of tumors. However, the antitumor activity of endostatin had not been studied previously in HNSCC models. We have previously established a method to produce recombinant endostatin from *E. coli* in a soluble form. We also showed that this soluble endostatin could significantly inhibit the growth of an immuno-
Combined Endostatin and EGFR-Antisense Therapy

**Fig. 5** Down-modulation of EGFR and VEGF by EGFR-antisense treatment *in vivo*. A, EGFR expression in tumor homogenates. Tumor nodules were resected at the experimental end point. The collected tumors were homogenized, and the supernatants were subjected to Western blot analysis for EGFR levels using a monoclonal antibody against human EGFR (BD Transduction Laboratory). The membrane was then stripped and reprobed with antibody against β-actin. The image was visualized by exposing to Kodak X-ray film. Lane 1, EGFR-sense; Lane 2, endostatin; Lanes 3–5, EGFR-antisense alone; Lanes 6–8, combination of EGFR-antisense and endostatin; Lane 9, lysate of cultured 1483 cells. B, VEGF levels in tumor homogenates. Supernatants obtained from the homogenized tumor samples were subjected to ELISA assay for VEGF (R&D System). One sample from the EGFR-sense control group (sense) or endostatin treatment group (endostatin) and two samples from the EGFR-antisense treatment group (antisense) or the combined therapy group (combined) were tested and shown. Each column was presented as the mean of triplicate ELISA wells; bars, SD.

One possible explanation might be that attacking the tumor vascular system solely by angiostatic monotherapy may not be sufficient to reverse tumor angiogenesis, which is supported by the tumor cells in a very aggressive and progressive manner. The robust ability of tumor cells to stimulate angiogenesis has been shown extensively in many studies. Tumor cells produce multiple highly active angiogenesis promoters that interact with one another to form an efficient angiogenic network (reviewed in Refs. 1, 23, and 24). Previous studies showed that even residual tumor cells surviving in a dormant state after endostatin therapy could strongly promote neovascularization and tumor growth, suggesting that the angiogenic activity of tumor cells impacts on the effect of antiangiogenic therapy (25). It is of note that typical antiangiogenic therapies, such as endostatin, mainly act against tumor endothelium and do not inhibit the production of angiogenic factors by tumor cells. Therefore, to deliver effective antiangiogenic cancer therapy, not only must the vascular components be attacked, but the angiogenic phenotype of the tumor cells should also be counteracted. In this context, combining an angiostatic drug that acts against the vascular endothelium with a strategy that targets the tumor cells might represent an efficient antiangiogenic approach. We combined endostatin and an EGFR-antisense construct and compared the antitumor and angiogenic effects of combined therapy with single-agent treatments. Our results demonstrated that the combination therapy resulted in nearly complete suppression of HNSCC growth in both xenograft and syngeneic models, whereas endostatin or EGFR-antisense alone led to partial inhibition of tumor growth. The complete inhibition of tumor growth...
growth by the combination treatment is compelling because we have shown that such an enhancement in therapeutic efficacy could not be reached by simply doubling the dose of either endostatin monotherapy or EGFR-antisense therapy (13). The biological significance of these results was further highlighted by the reproducibility of the therapeutic effect in two independent HNSCC models. These results suggest that EGFR-down-modulation strategies can be applied in combination with antiangiogenic endostatin to achieve high therapeutic efficacy.

EGFR signaling is known to be a critical stimulus for many tumor cell types. EGFR targeting therapies have been investigated extensively in preclinical and early phase clinical studies (5, 26, 27). Our in vivo experiments demonstrated significant, yet incomplete, inhibition of tumor growth delivered by EGFR-antisense treatment in HNSCC. The mechanism for the combined effect of endostatin and EGFR-antisense in this report is incompletely understood. The antiangiogenic effect of endostatin and the direct antitumor activity of EGFR-antisense treatment were additive to achieve a higher level of tumor suppression. Nonetheless, further analysis of the angiogenic status of the treated tumors showed clearly that tumor angiogenesis was completely blocked by the combination therapy, whereas each monotherapy merely led to partial inhibition of tumor angiogenesis. This result is consistent with the notion that solid tumors cannot grow beyond a microscopic size without an effective support of neovessels (1). These results suggest that a robust suppression of angiogenesis occurred under the combination treatment. Thus, in addition to its direct antiproliferative activity on tumor cells, EGFR down-modulation might also act cooperatively with endostatin to block tumor angiogenesis. Such an enhanced antiangiogenic effect might have contributed to the complete inhibition of the HNSCC tumors. Fidler’s group recently reported that inhibition of EGFR activity by EGFR-selective TKIs tremendously down-modulated the expression of VEGF and microvessel densities in pancreatic cancer, and that such an effect inhibited tumor growth cooperatively with antiangiogenic strategies (28, 29). On the basis of these studies, incorporation of EGFR targeting approaches in antiangiogenic cancer therapy might be a promising anticancer strategy.

The antiangiogenic effects of EGFR down-modulation have been shown previously (27, 30). Transforming growth factor-α and EGF were found to be angiogenic in vivo (31). However, the mechanism for the angiogenic effects of EGFR stimulation is not completely understood. Recent studies demonstrated that blockade of EGFR signaling by an EGFR-selective TKI inhibited angiogenesis and induced apoptosis of endothelial cells in vivo (28, 29). However, whether such an effect was mediated by a direct action on endothelial cells and/or indirectly via down-regulating angiogenic factors remains unclear. Our in vitro studies revealed the dual-effects of EGFR down-modulation on both cell compartments of HNSCC, inhibiting both the growth of endothelial cells and VEGF production by tumor cells. It was shown previously that EGFR stimulation up-regulated the expression of VEGF in tumor cells (9). Although EGFR expression on endothelial cells has been demonstrated (31, 32), the inhibitory effect of EGFR down-modulation on endothelial cell growth has not been studied extensively. Our results suggest that the inhibitory effects of EGFR targeting strategies on tumor angiogenesis might be a combined effect of both direct and indirect antiangiogenic functions.

In summary, we show that a combination of endostatin and EGFR-antisense gene therapy led to significantly enhanced inhibition of HNSCC growth in nude mice. The antitumor effect was mediated by both the antitumor effects of EGFR-antisense therapy and combined antiangiogenic effects of endostatin and EGFR-antisense therapy. Our data also indicate that EGFR targeting approaches directly suppressed endothelial cell growth and inhibited the angiogenic phenotype of HNSCC cells. Because both direct antiangiogenic modality and EGFR-targeting methods are currently under active clinical development, such a combination strategy might represent a novel and promising approach to HNSCC therapy.

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