Antiangiogenic Therapy of Established Tumors in Human Skin/Severe Combined Immunodeficiency Mouse Chimeras by Anti-Endoglin (CD105) Monoclonal Antibodies, and Synergy between Anti-Endoglin Antibody and Cyclophosphamide

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ABSTRACT

Endoglin (EDG; CD105) is a proliferation-associated cell membrane antigen of endothelial cells and is strongly expressed on the tumor-associated angiogenic vascular endothelium. Furthermore, EDG is essential for angiogenesis and is a component of the transforming growth factor (TGF)-β receptor complex. The present three anti-EDG monoclonal antibodies (mAbs), SN6f, SN6j, and SN6k, react strongly with proliferating human endothelial cells but cross-react very weakly with murine endothelial cells. Analysis of Scatchard plot of direct binding of these mAbs to proliferating human umbilical vein endothelial cells showed equilibrium constants of $8.3 \times 10^{-9}$, $3.1 \times 10^{-9}$, and $1.0 \times 10^{-9}$ liter/mol, respectively, for SN6f, SN6j, and SN6k. These mAbs did not react with MCF-7 human breast cancer cells. To facilitate antiangiogenic tumor therapy by these mAbs in animal models, we used human skin/severe combined immunodeficiency (SCID) mouse chimeras bearing tumors of MCF-7. Blood vessels in the chimeras were analyzed by immunostaining with species (human or mouse)-specific anti-CD31 and anti-EDG mAbs including an antihuman EDG mAb termed SN6h. Blood vessels in the completely healed grafted human skins consisted of a mixture of human (43.5%) and murine (56.5%) vessels, whereas only murine vessels were detected in the adjacent murine skins and s.c. tissues. Therefore, murine vessels infiltrated into the human skin grafts from the adjacent murine tissues, whereas the growth of human vessels is limited within the boundary of human skins.

Growth of human MCF-7 tumors in the human skin grafts increased the ratio of human:murine vessels. Analyses of the grafted skins before and after tumor transplantation showed that SN6h reacted with tumor-induced angiogenic blood vessels but not with nonangiogenic vessels, whereas antihuman CD31 mAb reacted with both angiogenic and nonangiogenic vessels. The results show that SN6h is capable of distinguishing the tumor-induced angiogenic vasculature from the nonangiogenic vasculature in the present model. Antiangiogenic therapy of the chimeras bearing established MCF-7 tumors was carried out by i.v. administration of a mAb(s) via the tail vein of mice. SN6j and SN6k were effective for suppressing the established tumors, whereas tumor suppression was weaker with SN6f. The results indicate an absence of a direct correlation of a mAb(s) via the tail vein of mice. SN6j and SN6k were effective for suppressing the established tumors, whereas tumor suppression was also effective for partial (i.e., 35%) suppression of murine vessels.

The results show that systemic administration of naked anti-EDG mAbs can suppress established tumors, and the efficacy is markedly enhanced by combining a chemotherapeutic drug using an antiangiogenic schedule of drug dosing. These mAbs should show stronger antitumor efficacy in patients whose tumors depend entirely on human blood vessels.

INTRODUCTION

A homodimer glycoprotein antigen, later termed EDG (CD105), was initially identified as a human leukemia-associated cell membrane antigen (1, 2). Its expression is restricted to immature B-lineage acute lymphoblastic leukemia cells, myelomonocytic leukemia cells, endothelial cells, and a few minor normal cells (1–5). EDG binds TGF-β1 and TGF-β3 specifically but fails to bind TGF-β2 (6). The role of EDG in the TGF-β-induced signal transduction is poorly understood. EDG is a proliferation-associated antigen on leukemia cells (7) and endothelial cells (8–10). Furthermore, EDG is essential for angiogenesis (11). Certain anti-EDG mAbs react with tumor-associated vascular endothelium more strongly than with vascular endothelium in normal tissues (8, 12–15). Therefore, these selected anti-EDG mAbs and their immunoconjugates may be useful for antiangiogenic therapy by targeting EDG on tumor-associated vascular endothelium. Recently, we showed that immunoconjugates of selected anti-EDG mAbs that weakly cross-react with mouse endothelial cells were effective for suppressing angiogenesis and tumors in mice (9, 15, 16); these mAbs correspond to externally induced autoantibodies (17). Anti-EDG mAbs were conjugated either with deglycosylated ricin A-chain to obtain immunotoxins (9, 15) or with $^{125}$I to obtain radioimmunoconjugates (16). Although the anti-EDG immunotoxins showed strong antiangiogenic antitumor efficacy at the dose of 24–45% of the LD$_{50}$, they showed strong toxicity in mice; LD$_{50}$ of SN6f, SN6j, and SN6k immunotoxins ranged between 14.8 and 17.8 µg/g of body weight (9, 15). Therefore, the therapeutic windows of these immunotoxins are relatively narrow. In contrast to immunotoxins, naked anti-EDG mAbs showed no significant toxicity in a dose-escalation study in mice (see “Discussion”). Relevant observation was reported for anti-HER2 immunotoxin and mAbs. An anti-HER2 immunotoxin showed a strong hepatotoxicity in patients because hepatocytes express HER2 antigen (18). Nevertheless, the toxicity of anti-HER2 mAb was weak in patients (19, 20) and anti-HER2 mAb has been widely used in patients, particularly in the combination therapy with a chemotherapeutic drug (e.g., Ref. 21).

In the present study, we investigated antitumor efficacy of naked (unconjugated) anti-EDG mAbs. A disadvantage of a naked anti-EDG

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3 The abbreviations used are: EDG, endoglin; SCID, severe combined immunodeficiency; mAb, monoclonal antibody; TGF, transforming growth factor; HUVEC, human umbilical vein endothelial cell; CPA, cyclophosphamide; dgRA, deglycosylated ricin A chain; i.d., intradermal/intradermally.
mAb, compared with an immunoconjugate, is weaker antitumor efficacy. To circumvent this problem, we used human skin/SCID mouse chimeras (22–24) in the present study. Rationale for using the chimera is as follows. The newly formed blood vessels in the transplanted human tumors in the immunodeficient animals originate from the host’s tissues. Our anti-EDG mAbs cross-react very weakly with mouse endothelial cells, whereas they react much more strongly with human endothelial cells (9, 15). The newly formed blood vessels in the transplanted human tumors in human skin grafted to SCID mice consist of human and mouse vessels (see below). Therefore, our anti-EDG mAbs will be more effective for antiangiogenic tumor therapy in human skin/SCID mouse chimeras than in SCID mice. Our results support to assert this theory. Furthermore, the combination of an anti-EDG mAb with CPA showed synergistic antitumor efficacy.

MATERIALS AND METHODS
mAbs and Reagents. Antihuman EDG mAbs SN6f (termed K4–2C10), SN6j, and SN6k that cross-react weakly with mouse endothelial cells were generated in our laboratory (9, 15). A non-cross-reactive anti-EDG mAb, SN6h (14, 25), and an isotype-matched murine control IgG (MOPC195 variant, IgG1-k; Ref. 26) were also generated in our laboratory. Murine antihuman CD31 mAb JC70A (IgG1-k) and CPA were obtained from DAKO (Carpinteria, CA) and Sigma Chemical Co. (St. Louis, MO), respectively. Rat antimouse EDG (CD105) mAb MJ7/18 (rat IgG2a-k), rat antimouse CD31 mAb 390 (rat IgG2a-k) and an isotype-matched rat control IgG (R35–95; IgG2a-k) were purchased from PharMingen (San Diego, CA). InnoGenex Mouse-on-Mouse Iso-IHC kit, peroxidase substrate kit DAB (SK-4100), and LSAB kit Peroxidase Universal (K568) were purchased from InnoGenex (San Ramon, CA), VECTOR (Burlingame, CA), and DAKO, respectively.

Tissues, Cells, and Mice. Fresh human neonatal foreskins from elective circumcision were obtained from the Cooperative Human Tissue Network (CHTN) Eastern Division, University of Pennsylvania Medical Center. MCF-7 human breast cancer cells, KM-3 human leukemia cells, HUVECs, and SVEC4–10 murine endothelial cells (27) were cultured as described previously (9). Female SCID (Ncr) mice were obtained from the Frederick Cancer Research and Developmental Center, National Cancer Institute (Frederick, MD). Mice were maintained in a protected environment in a laminar flow unit and given sterilized food and water ad libitum as described previously (28). All of the handling of SCID mice was performed in a laminar flow hood.

Antigen-binding Avidity of mAbs. The purified mAbs SN6f, SN6j, and SN6k were individually radiolabeled with 125I using Iodo-Gen as described previously (1). The radiolabeled SN6f, SN6j, and SN6k were determined to contain 1.85, 0.73, and 1.29 iodine atoms per IgG molecule on the average, respectively. Titration experiments which were carried out using a fixed amount (0.1 μg) of each 125I-labeled mAb and 2-fold serial increments of EDG-expressing KM-3 cells showed that 48.6, 50.0, and 55.0%, respectively, of the labeled SN6f, SN6j, and SN6k retained antigen-binding activity. In the analyses of the binding data for SN6f, SN6j, and SN6k, corrections were made for the above numbers. An analysis of Scatchard plot of binding data was carried out as described previously (29, 30). An equilibrium constant and an average maximal number of mAbs bound per cell were estimated by this analysis.

Cellular RIA. An indirect cellular RIA was used to determine the reactivities of antihuman EDG mAbs with murine endothelial cells. Details of the assay were described previously (31). Briefly, mAbs and an isotype-matched control IgG were individually incubated, in triplicate, with target cells in wells of 96-well microtiter plates at 4°C for varying periods of time. After the cells were pelleted and washed three times, 125I-labeled F(ab’), of affinity-purified goat antimouse IgG antibodies was added to the cells, and the reaction mixtures were incubated at 4°C for 1 h. After the cells were washed four times, the radioactivity in the washed cells was determined in a Cobra Series Autogamma Counter (Packard Instrument Comp., Meriden, CT).

Chimeric Human/Mouse Model. Fresh human neonatal foreskins (see above) were stored in sterile RPMI 1640 supplemented with 2% fetal bovine serum, 1% gentamicin, and 2.5 μg/ml fungizone. The tissues were used for grafting on the same day as received. The grafting into SCID mice was performed as described by others (22–24) with slight modifications. The procedures are briefly described below.

Female SCID mice (7–10 weeks old) were anesthetized, and a section of skin (~2 cm2) was surgically removed. A precut section of fresh, full-thickness, human neonatal foreskin was sutured into place. The grafts were bandaged securely for 2 weeks to allow healing. EDG-negative MCF-7 cells were inoculated i.d. into the grafted human skins over 7 weeks after the skins were grafted, and the graft healed completely.

Immunostaining of Tissues. Serial sections (6 μm in thickness) were cut from the frozen tissues using a cryostat, fixed in cold acetone, and stored at −70°C until use. Human and mouse blood vessels in the tissue slice were determined by immunohistochemical staining using species-specific antendothelial mAbs, i.e., mouse antihuman EDG mAb (SN6h), mouse antihuman CD31 mAb, rat antimouse EDG (CD105) mAb, and rat antimouse CD31 mAb. In the immunostaining, isotype-matched control murine IgG (MOPC 195 variant; IgG1-k) and isotype-matched control rat IgG (R35–95; IgG2a-k) were included as controls. For immunostaining of mouse tissues using mouse mAbs, InnoGenex Mouse-on-Mouse Iso-IHC kit and Vector peroxidase substrate kit DAB were used following the manufacturer’s instructions to reduce background staining. For immunostaining of mouse tissues using rat mAbs, DAKO LSAB Kit Peroxidase Universal was used. Counterstaining was performed with hematoxylin. The ratio of human/mouse vessels in the completely healed human skin grafts (i.e., 8 weeks after the grafting), and in the human skin grafts bearing MCF-7 tumors, was determined by counting human and mouse vessels on four ×100 fields (i.e., ×10 objective lens and ×10 ocular lens) at the area of highest vascularization (32) in each tissue section under a microscope. Serial sections of the human skins were stained with a mixture of SN6h and antihuman CD31 mAb, and a mixture of antihuman CD105 mAb and antimonocytic CD31 mAb.

In addition, we immunostained mouse and human vessels in the tumors from the chimeric mice that received PBS (control), SN6f, CPA, or SN6j plus CPA, and were killed at the end of a therapeutic experiment. Large tumors of two different sizes (i.e., 350–450 mm3 and 100–250 mm3) were obtained from each group of mice for immunostaining and vessel counting. Sections of the tumors were stained with a mixture of antihuman CD105 and CD31 mAbs and a mixture of antihuman CD105 and CD31 mAbs as described above. Human and mouse vessels were counted separately as described above. An average of vessel counts in the two tumors was compared between different groups.

Antiangiogenic Therapy of Preformed Human Tumors in Human Skins Grafted into SCID Mice. MCF-7 cells (8 × 105 cells in 0.1 ml of PBS) were transplanted i.d. into human, full-thickness, skins grafted into SCID mice when the grafts showed no signs of inflammation, contraction, or rejection. The mice were left untreated until distinct palpable tumors (3 to 6 mm in diameter in most cases) appeared. Mice with distinct tumors were divided into groups for the therapeutic studies. Anti-EDG mAbs and an isotype-matched control IgG (MOPC 195 variant; IgG1-k) were centrifuged at 100,000 × g for 1 h, and the supernatants were individually filtered through a sterile Millex-GV filter (0.22 μm; Millipore, Bedford, MA) in a laminar flow hood before use. The sterilized solutions were diluted with sterile PBS containing mouse serum albumin (0.05% final concentration). For the mAb therapy, 200 μg/0.2 ml mAb or control IgG was administered i.v. via the tail vein of mice. The administration was repeated four times every 2 days. After a break for a week, the second cycle of the therapy was performed as described for the first cycle. For the combination therapy with CPA, 200 μg mAb and CPA (80 mg/kg of body weight) were given i.v. and i.p., respectively, mAbs were given i.v. for systemic therapy of tumors. However, CPA was given i.p. because CPA is a small molecule (i.e., M, 279.1) and would be rapidly eliminated from circulation when mice receive it by bolus i.v. injections. CPA was given following an antiangiogenic schedule (33, 34). Administration of the mAb and CPA was repeated as indicated in the corresponding figures in “Results.” Three control groups received PBS, mAb, and CPA, respectively.

Follow-Up of Treatment Efficacy. During the treatment, mice were monitored daily for tumor and morbidity. Mice were weighed twice a week using an electronic balance (OH-AUS Model GT210). Tumor size was measured twice or three times a week using an electronic caliper (PRO-MAX 6 inch caliper; Fowler Co., Newton, MA) that was connected to a computer using OptoDemo software (Fowler Co.). The measured tumor diameters were con-
verted to tumor volumes using Excel 97; the tumor volumes were calculated using the following formula (35):

\[ V = \text{Length} \times \text{width} \times \text{height} \times \frac{\pi}{6} \]

Statistical analysis of the data for the comparison of different groups of mice was carried out using Student’s t test.

RESULTS

Immunostaining of Blood Vessels in Tissues from Human Skin/SCID Mouse Chimeras. Vessels in the human skin grafts and the adjacent mouse tissues were analyzed by immunohistochemical staining with species-specific antiendothelial mAbs, i.e., SN6h (an antihuman CD105 mAb), antihuman CD31 mAb, antimouse CD105 mAb, and antimouse CD31 mAb. The ratio of human vessels to mouse vessels in the completely healed human skin grafts and in the skin grafts bearing large MCF-7 tumors was determined in serial sections of the tissues by counting human vessels and mouse vessels. Human vessels were detected by immunostaining the tissue sections with a mixture of SN6h and antihuman CD31 mAb, and mouse vessels were detected by immunostaining with a mixture of antimonuse CD105 mAb and antimouse CD31 mAb. Blood vessels in the completely healed (8 weeks after grafting) human skin without tumors consisted of 43.5% human vessels and 56.5% mouse vessels. Blood vessels in the human skin bearing a large established human tumor (250 mm\(^2\)) consisted of 59.6% human vessels and 40.4% mouse vessels. Therefore, growth of tumors in the human skin grafts depended on both murine and human vessels. The results also indicate that human tumors stimulate human vessels preferentially, compared with murine vessels. Immunostaining of vessels was also performed using individual mAbs. The stained vessels in the human skin grafts and adjacent mouse tissues are illustrated in Fig. 1, A–D. In addition, vessels in MCF-7 tumors in the grafted human skin and the adjacent s.c. murine tissues are illustrated in Fig. 1, E and F). SN6h did not stain any vessels in the grafted human skins or adjacent mouse skins (Fig. 1A). However, SN6h stained multiple vessels in tumors in the human skins but not in the adjacent murine s.c. tissues (Fig. 1E). The results show that SN6h is capable of distinguishing the tumor-induced angiogenic vessels from the nonangiogenic vessels. In contrast, antihuman CD31 mAbs stained vessels in the tumor-free human skins (Fig. 1B) and also vessels in the tumors in the grafted human skins (data not shown). It did not stain any vessels in the adjacent murine skins (Fig. 1B). Antimouse CD105 mAbs stained vessels in the human skins as well as in the adjacent murine skins (Fig. 1C). It also stained vessels in MCF-7 tumors in the human skins and vessels in the adjacent murine s.c. tissues (Fig. 1F). Thus, unlike SN6h, the antimonuse CD105 mAb was unable to distinguish the nonangiogenic vessels from the tumor-induced angiogenic vessels. The staining pattern of antimonuse CD31 mAb is similar to that of antimouse CD105 mAb; the former stained vessels in the human skins as well as in the adjacent murine skins (Fig. 1D). The described immunostaining pattern of human skin grafts and the adjacent mouse tissues was consistently observed for the tissue samples obtained 7, 8, 9, 11, 18, and 20 weeks after grafting of the human skins. Specificity of the antimonuse CD105 and antimouse CD31 mAbs for murine vessels was confirmed by immunohistochemical studies in which these mAbs did not react with any blood vessels in human colon cancer tissues and human neonatal foreskins. The results show that the growth of human vessels is limited within the boundary of the grafted human skins, but murine vessels infiltrate into the grafted human skins from the surrounding murine tissues. Therefore, murine vessels will be more functional for delivering nutrients and oxygen than human vessels in the human skin grafts.

Antibody Avidity and Number of Available Epitopes on EDG-expressing Cells. Scatchard plot analyses of direct binding of radio-labeled SN6f, SN6j, and SN6k to EDG-targeted antiangiogenic therapy

EDG-TARGETED ANTIANGIOGENIC THERAPY

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effective for tumor suppression compared with 400 μg of an isotype-matched control IgG. A similar test was performed with SN6j (Fig. 5). Both 200 μg and 400 μg of SN6j were effective for tumor suppression compared with the isotype-matched control IgG. The difference in the antitumor efficacy between 200 μg and 400 μg of SN6j was small. The results show that naked anti-EDG mAbs can suppress growth of established tumors.

Synergistic Antitumor Efficacy by Combination of a mAb with a Chemotherapeutic Drug. To improve antitumor efficacy of the naked anti-EDG mAb, SN6j was combined with CPA that was administered i.p. into tumor-bearing chimeras using an antiangiogenic schedule of drug dosing (33, 34) or, in another term, a metronomic dosing regimen (36). MCF-7 tumors in human skin grafts in SCID mice were established as described above. Chimeras with established
tumors were distributed into four groups \( n = 11005 \). Individual groups were treated by systemic administration of PBS, SN6j, CPA, or SN6j plus CPA. The results are presented in Fig. 6. It should be noted that at the onset of therapy, the average size of tumors in the present set of experiments is larger than those presented in Figs. 3, 4, and 5; the average size of tumors was 63.8, 57.5, 54.6, and 97.2 mm\(^3\), respectively, for Figs. 3, 4, 5, and 6. Both SN6j and CPA are effective for tumor suppression. Difference in the tumor size between the control PBS group and the SN6j/CPA-treated group is statistically significant \( P < 0.04 \) after day 45 and until the end of experiment, i.e., day 66. The potential synergy between two antitumor agents was evaluated as described by others (e.g., Ref. 37). Table 1 summarizes relative tumor volume of control and treated groups on five different time points during the therapy. Combination therapy showed more than additive effect on tumor growth suppression. On day 24, 9 days after therapy initiation, there was a slight improvement in antitumor activity in the combination group when compared with the expected additive effect. This improvement became greater during the course of therapy. On days 34 and 45, there were 1.5-fold and 1.6-fold improvements, respectively, in the combination group compared with the expected additive effect (Table 1). This supraadditive effect of the combination therapy persisted until the end of the experiment (i.e., day 66). The results demonstrate that SN6j and CPA exert synergistic antitumor efficacy in the present model. Small decreases in the body weight were observed for the chimeras treated with CPA and CPA plus SN6j; the decreases were within 10% of the control. Therapeutic effect on individual chimeras in each group of Fig. 6 is presented in Fig. 7.

Fig. 2. Binding of \(^{125}\)I-labeled SN6f, SN6j, and SN6k to EDG-expressing KM-3 cells (A) and HUVECs (B). \( r \) is the number of antibody molecules bound to one cell at a given dilution, \( A \) is the molar concentration of total antibody, \( x \) is the molar concentration of bound antibody, so that \( (A - x) \) is the molar concentration of the free antibody. The slope of the binding curve gives the equilibrium constant, \( K \). The intersection point between the binding curve and abscissa gives the maximum number of antibody molecules, \( n \), that can be bound to a single cell. The equilibrium constant for the reaction, \( K \), is expressed in liters per mole.

Fig. 3. Therapy of established tumors in human skin/SCID mouse chimeras by i.v. administration of naked anti-EDG mAbs. MCF-7 human breast cancer cells were injected into human skin grafts in SCID mice, and therapy was delayed until distinct palpable tumors appeared. Chimeras with distinct tumors were divided into groups \( n = 6 \) except for PBS \( n = 5 \) and MOPC \( n = 5 \) groups) and treated with PBS, an isotype-matched control IgG (MOPC), SN6f, SN6j, SN6k, or SN6f plus SN6k. The therapy was performed in two cycles including 1-week pause between the cycles to relieve the mice from the i.v. injection-associated stress. Each cycle consisted of five injections, at 2-days intervals, of PBS, a control IgG (200 \( \mu \)g), a mAb (200 \( \mu \)g), or a mixture of SN6f (200 \( \mu \)g) and SN6k (200 \( \mu \)g) as indicated in the figure. Arrows in the figure, injections of PBS, a control IgG, a mAb, or a mixture of two mAbs.

Fig. 4. Effect of different doses of SN6k on the antitumor efficacy. Established tumors in human skin/SCID mouse chimeras were generated as in Fig. 3. Groups of chimeras \( n = 6 \) in each group) were treated by i.v. administration of an isotype-matched control IgG (400 \( \mu \)g), 200 \( \mu \)g SN6k, or 400 \( \mu \)g SN6k. Injections of the control IgG and SN6k were repeated as in Fig. 3.

Fig. 5. Therapy of preformed tumors by systemic administration of different doses of SN6j. Three groups of chimeras bearing established tumors \( n = 6 \) were treated by i.v. administration of 400 \( \mu \)g of an isotype-matched control IgG, 200 \( \mu \)g SN6j, or 400 \( \mu \)g SN6j. Arrows, injections of control IgG or SN6j.
Growth rate of MCF-7 tumors in the human skin/SCID mouse chimeras was substantially heterogeneous before the onset of the therapy. This is in contrast to the more homogeneous growth of the MCF-7 tumors in SCID mice (e.g., Ref. 15). The major reason for this heterogeneous growth in the chimeras may be the wider genetic heterogeneity of the human skins compared with SCID mice. To minimize the effect of the heterogeneous tumor sizes on the experiment, chimeras with tumors of a similar size were distributed nearly evenly into different groups. Consequently, the average size of tumors in each group became similar among the four groups at the onset of therapy (see Fig. 6). Tumors in 8 chimeras of the control PBS group all continued growing, although the growth rate of one tumor was substantially slower than others (Fig. 7A). Treatment with SN6j slowed tumor growth compared with the control group, and a small tumor in one chimaera regressed completely (Fig. 7B). CPA therapy using an antiangiogenic schedule slowed the growth of five smaller established tumors and induced near regression of one of the tumors. However, CPA therapy showed little therapeutic effect on the three large tumors of more than 100 mm³ at the onset of therapy (Fig. 7C). Treatment with SN6j and CPA using an antiangiogenic schedule of drug dosing prevented five smaller established tumors from increasing in size and induced lasting complete regression of two of the tumors (Fig. 7D). An additional tumor regressed after day 37 but relapsed soon after the regression. The combination therapy prevented one of the three large tumors of more than 100 mm³ from increasing in size soon after the initiation of therapy, and the tumor remained stable during and after the therapy (Fig. 7D). Two of the large tumors continued growing initially but became stable after three injections of SN6j and CPA; these tumors restarted expanding after cessation of therapy. The present results show that the combination of SN6j and CPA is most effective for treating both small and large established tumors. The results also indicate that the initial size of the established tumors is an important factor for the outcome of the therapy. The anti-EDG mAbs, and the combination of an anti-EDG mAb and CPA should be much more effective for tumor suppression in patients than in the human skin/SCID mouse chimeras (see “Discussion”).

We immunostained human and murine blood vessels in the large human tumors from the chimera mice after the mice were killed at the end of experiment shown in Fig. 7 (see “Materials and Methods” for details). Number of murine vessels in the tumors was 52, 47, 47, and 34 per 100 × field for groups A, B, C, and D, respectively (see Fig. 7 for the groups). Therefore, SN6j (group B) and CPA (group C) caused only a small decrease (~10%) in the number of murine vessels in the tumors compared with the control (group A). However, combination of SN6j and CPA (group D) caused a larger (i.e., 35%) decrease in the number of murine vessels. The number of human vessels in the tumors was 33, 0, 36, and 0 per 100 × field for groups A, B, C and D. Therefore, SN6j completely suppressed human vessels in the tumors, whereas CPA was not effective for suppressing human vessels in the tumors. The results show that SN6j is highly effective for suppressing human vessels but only weakly suppressive against murine vessels. CPA was not effective for inhibiting human vessels and only weakly suppressive against murine vessels. However, combination of SN6j and CPA showed a stronger suppression of murine vessels and was effective in eliminating human vessels in the tumors.

We would like to add a cautionary note that the tumors used in the present vessel counting were obtained from chimera mice whose therapy was terminated 3 weeks before the mice were killed and tumors were resected. Therefore, the status of murine and human vessels in these tumors may not be the same as that in the tumors from mice receiving therapy. Nevertheless, the present test reveals different effects of SN6j, CPA, and SN6j plus CPA on tumor vasculature.

**DISCUSSION**

Antiangiogenic therapy of cancer is highly attractive for several reasons (reviewed in Refs. 38–40). For instance, it can potentially overcome three major problems associated with other anticancer therapies, i.e., the problems of drug resistance (41, 42), poor delivery (43, 44), and tumor heterogeneity. One approach to the antiangiogenic therapy is antibody-based targeting of tumor vasculature. We have been targeting EDG on tumor vasculature in vivo (9, 15, 16). Several features of EDG are described above (see “Introduction”). In addition, our anti-EDG mAbs showed little reactivity with normal human bone marrow cells (1). This observation is consistent with our later finding that anti-EDG immunotoxin (i.e., ricin A-chain conjugate of SN6) selectively eradicated EDG-expressing cells without severely damaging normal human hematopoietic progenitors in the colony-forming unit assays. The results suggest that administration of therapeutic doses of an appropriate anti-EDG mAb and immunotoxin may not significantly damage hematopoietic progenitors in patients. Nevertheless, anti-EDG immunotoxins showed significant toxicity in mice (see

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**Table 1 Combination therapy with SN6j and CPA**

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<th>Day*</th>
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<th>CPA</th>
<th>Expected†</th>
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<th>Ratio of expected:observed FTV‡</th>
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* Day after tumor cell inoculation. Tumor volume was measured twice or three times a week for each mouse (n = 8 for each group of mice).
† FTV, calculated as mean tumor volume experimental/mean tumor volume control. CPA was not effective for inhibiting human vessels but only weakly suppressive against murine vessels. However, combination of SN6j and CPA showed a stronger suppression of murine vessels and was effective in eliminating human vessels in the tumors.
‡ Obtained by dividing the expected FTV by the observed FTV. A ratio of >1 indicates a synergistic effect, and a ratio of ≤1 indicates a less than additive effect.

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**Fig. 6.** Improved antitumor efficacy by the combination of an anti-EDG mAb with a chemotherapeutic drug and by using an antiangiogenic schedule of drug dosing. Established tumors of MCF-7 in human skin/SCID mouse chimeras were generated as described in Figs. 3, 4, and 5. Groups of chimeras bearing distinct tumors (n = 8 in each group) were treated by administration of PBS, SN6j, CPA, or SN6j plus CPA. CPA (80 mg/kg body weight) was injected i.p. every 4 days by using an antiangiogenic schedule (33, 34). SN6j (200 μg) was administered i.v. every 3 days for the first five injections and every 4 days for the remaining five injections. *, difference in the tumor size between the PBS group and the SN6j + CPA group is statistically significant (P < 0.04) after day 45 and until the end of the experiment. Solid arrows, injections of SN6j; dashed arrows, injections of CPA.
should be noted that injections of PBS in A of SN6j; previously, Juhasz healed human skin grafts 7 or more weeks after the skin was transplanted. We inoculated MCF-7 human breast cancer cells into the completely destroyed murine vessels during growth of inoculated tumors. The present results demonstrate that human skin grafts contain blood vessels in several tissue samples from human skin/SCID mouse chimeras whose human skins had been transplanted 7, 8, 9, 11, 18, and 20 weeks earlier. We used antihuman CD31 mAb and antimouse CD31 mAb, in addition to antihuman CD31 polyclonal antibodies. In the immunostaining, they used human skin that had been transplanted 4 weeks earlier. They detected only human vessels in human skin and only mouse vessels in the adjacent mouse skin. We immunostained blood vessels in several tissue samples from human skin/SCID mouse chimeras whose human skins had been transplanted 7, 8, 9, 11, 18, and 20 weeks earlier. We used antihuman CD105 mAb and SN6h, an antimouse CD105 mAb, in addition to antimouse CD31 and antihuman CD31 mAbs in the immunostaining. All of these mAbs are species specific (e.g., see "Results"). Both antihuman CD105 mAb and antimouse CD31 mAb consistently stained vessels in human skins that had been transplanted 7–20 weeks earlier (e.g., see Fig. 1, C and D). Antihuman CD31 mAb stained vessels in human skins but not in adjacent murine skins. These results, showing restricted growth of human vessels, are consistent with the observation of Yan et al. (22). SN6h stained only tumor-induced angiogenic human vessels (see Fig. 1). Results of Yan et al. (22) and present studies suggest that the infiltrating murine vessels in human skin grafts become immunohistochemically detectable between 4 and 7 weeks after transplantation of human skins. Alternatively, the observed difference in the infiltrating murine vessels may be attributable to difference in the sensitivity of the immunostaining reagents and/or methods. Despite these questions, the present results demonstrate that human skin grafts contain both human and murine vessels during growth of inoculated tumors. We inoculated MCF-7 human breast cancer cells into the completely healed human skin grafts 7 or more weeks after the skin was transplanted. Previously, Juhasz et al. (23) and Brooks et al. (24) successfully used the chimeras to grow human melanomas and human breast cancer, respectively. In view of the observation that the growth of human vessels was limited to remain within the boundary of human skin and that murine vessels infiltrated into human skin from the surrounding murine tissues, murine vessels will be more functional for delivering nutrients and oxygen than human vessels in the human skin grafts.

Growth rate of MCF-7 tumors in the chimeras is more heterogeneous than that of MCF-7 tumors in SCID mice (e.g., Ref. 15). The heterogeneous growth pattern of human tumors in the chimeras appears to represent tumor growth in patients better than the homogeneously growing tumors in immunodeficient mice. The major reason for this heterogeneity in the chimeras may be attributable to the genetic heterogeneity of the human skins compared with SCID mice used for tumor inoculation. Another reason may be that the time interval between skin grafting and tumor inoculation was variable. This variation occurred because the supply of human skins was scarce and unpredictable. Therefore, a substantial period of time was needed to accumulate enough chimeras for starting an experiment in which the grafted human skins were inoculated with tumor cells.

Despite this problem of heterogeneity, systemic (i.v.) administration of naked anti-EDG mAbs SN6j and SN6k consistently showed significant antitumor efficacy compared with an isotype-matched control IgG and/or PBS. The results showed that naked anti-EDG mAbs can suppress established tumors. SN6f that showed the highest antigen-binding avidity among the three anti-EDG mAbs was less effective for tumor suppression than were SN6j and SN6k. These results are consistent with our earlier observation that SN6j-dgRA and SN6k-dgRA were more effective for suppressing established tumors than was SN6f-dgRA (15). The results indicate an absence of a direct correlation between antigen-binding avidity and in vivo antitumor efficacy for anti-EDG mAbs. Although antigen-binding avidity of a mAb may still be an important factor for antitumor efficacy, other factors such as epitope and specificity may also be important determinants involved in antitumor efficacy. SN6f defines an epitope distinct from those defined by SN6j and SN6k (15). Our recent study revealed that SN6f epitope resides in peptide 250–305 of EDG.

Fig. 7. Tumors in individual human skin/SCID mouse chimeras that were treated with PBS, SN6j, CPA, or SN6j plus CPA. Growth rate of MCF-7 tumors in human skin grafts in individual SCID mice varied substantially before initiation of the therapy. Therefore, chimeras with tumors of a similar size were distributed nearly evenly into different groups at the onset of therapy. Consequently, the average size of tumors in each group became similar at the onset of therapy (see Fig. 6). Arrows, injections of PBS; solid arrows, injections of SN6j; dashed arrows, injections of CPA. It should be noted that injections of PBS in A were performed i.v. (upper line) and i.p. (lower line) as controls of SN6j and CPA, respectively. PBS was injected i.p. as a control of CPA in B, and it was injected i.v. as a control of SN6j in C.

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molecule, whereas SN6j epitope is in peptide 204–250. The molecular location of SN6k epitope remains to be determined. Concerning specificity of the mAbs, SN6j and SN6k showed selective reactivity with human tumor vasculature compared with normal human tissue vasculature (15). This selectivity was less distinct for SN6f.7

Although expression of EDG is highly restricted among different tissues and cells in vivo and certain anti-EDG mAbs react with tumor-associated vascular endothelium more strongly than with vascular endothelium in normal tissues (see “Introduction”), EDG is not a tumor-specific marker and EDG is expressed in varying degrees in the vasculature of normal tissues. Despite this limitation, we could effectively target tumor-associated vasculature using selected anti-EDG mAbs and immunoconjugates (9, 15, 16, and this report). We believe that this effective targeting of tumor-associated vascular endothelium is attributable to the combined effect of the following: (a) certain anti-EDG mAbs show a highly restricted reactivity among different tissues and cells; (b) EDG is a proliferation-associated antigen on endothelial cells and its expression is up-regulated in tumor-associated vascular endothelium (8–10, 12–15); and (c) turnover of endothelial cells of normal adult tissue vasculature is very slow (e.g., more than 1,000 days), whereas these endothelial cells undergo rapid proliferation during spurts of angiogenesis in tumors (39, 45, 46).

Therefore, the rapidly dividing endothelial cells of tumor vasculature are much more susceptible to killing by anti-EDG mAbs and immunoconjugates than the quiescent vascular endothelium of normal tissues. In addition, other undefined factors may also be involved.

In the present study, relatively large quantities of naked anti-EDG mAbs were used for treating tumors. Recently, however, we found that i.v. administration of a much smaller dose (i.e., three injections of 17 μg each) of naked SN6j and SN6k was effective for suppressing metastasis and primary tumors in a syngenic tumor model of immunocompetent mice, i.e., BALB/c mice bearing murine colon-26 tumors. The results suggest the importance of an immune status of hosts in antibody therapy of cancer. These anti-EDG mAbs should exert much stronger antitumor efficacy in patients than in the chimeras because tumors in patients will entirely depend on human blood vessels, and the vessels in patients are expected to be more functional for delivering nutrients and oxygen than human vessels in the human skin grafts, of which growth is limited within the boundary of the skin grafts. It should be noted that these anti-EDG mAbs react much more strongly with human vessels than with murine vessels. For this reason, however, these mAbs may show a stronger undesirable toxicity in patients than in mice. This question needs to be addressed before these mAbs are applied for therapy in patients.

Several investigators reported that the combination of an antiangiogenic agent with other cytotoxic agents or with ionizing radiation improved therapeutic efficacy compared with the antiangiogenic agent alone (e.g., Refs. 33, 34, 47–49). We are particularly interested in the combination of an anti-EDG mAb with a chemotherapeutic drug because such a combination can be readily tested in our study. Recently, Browder et al. (33) and Klement et al. (34) reported that the combination of an antiangiogenic agent with a chemotherapeutic drug using anti-angiogenic schedules of drug dosing showed marked antiangiogenic tumor suppression. Furthermore, a drug given following the antiangiogenic schedules could obviate the problem of acquired drug resistance (33). In view of these reports, we combined anti-EDG mAb SN6j with CPA/an antiangiogenic schedule in the therapy of established tumors in the chimeras. The combination therapy showed synergistic antitumor efficacy (Fig. 6; Table 1). No strong toxicity was observed by the combination therapy, although transient decreases in the body weight (<10%) of the chimeras were detected.

The results suggest superiority of the combination therapy over a single-agent therapy, either with CPA or SN6j alone. These results appear to warrant additional studies of the combination therapy.

Effects of TGF-β on cancer are complex (reviewed in Refs. 50–53). Although TGF-β is a potent growth inhibitor in epithelial tissues, it is both a suppressor and a promoter of tumorigenesis. Recently, Ananth et al. (54) reported that the neutralizing antibody against TGF-β inhibited tumorigenesis and, in some cases, regressed established 786–0 renal cell carcinoma tumors in athymic mice. On the other hand, Gohongi et al. (55) reported that treatment with neutralizing antibody against TGF-β reversed both angiogenesis suppression and inhibition of leukocyte rolling induced by gallbladder tumors in SCID mice. Our recent studies suggest that multiple mechanisms are involved in the suppression of tumors by naked anti-EDG mAbs. However, the mechanisms remain to be defined further.

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Antiangiogenic Therapy of Established Tumors in Human Skin/Severe Combined Immunodeficiency Mouse Chimeras by Anti-Endoglin (CD105) Monoclonal Antibodies, and Synergy between Anti-Endoglin Antibody and Cyclophosphamide

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