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 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 × 10⁹, 3.1 × 10⁹, and 1.0 × 10⁹ 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 infiltrate 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 Cyclophosphamide. This article must therefore be hereby marked in accordance with 18 U.S.C. Section 1743 solely to indicate this fact.

INTRODUCTION

A homodimer glycoprotein antigen, later termed EDG³ (CD105), was initially identified as a human leukemia-associated cell membrane antigen (1, 2). Its expression is restricted to immature 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 immunotoxins may be useful for antiangiogenic therapy by targeting EDG on tumor-associated vascular endothelium. Recently, we showed that immunotoxins 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 with¹²⁵I to obtain radioimmunoconjugates (18). Although the anti-EDG immunotoxins showed strong antiangiogenic antitumor efficacy at the dose of 24–45% of the LC₅₀, they showed strong toxicity in mice; LC₅₀ 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 mAb or immunotoxin is its stability in vivo. To overcome this disadvantage, we used two types of immunoconjugates (16). Although the anti-EDG immunotoxins showed strong antiangiogenic antitumor efficacy at the dose of 24–45% of the LC₅₀, they showed strong toxicity in mice; LC₅₀ 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).

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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 chimeras 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 appear to support this theory. Furthermore, the combination of an anti-EDG mAb with CPA showed synergistic antitumor efficacy.

MATERIALS AND METHODS

mAbs and Reagents. Anti-EDG human 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 anti-human CD31 mAb JC70a (IgG1-κ) 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–κ), rat antimonoc jurisdiction CD31 mAb 390 (rat IgG2a-κ) and an isotype-matched control rat IgG (R35–95; IgG2a-k) were purchased from Pharmingen (San Diego, CA), InnoGenex Mouse-on-Mouse Iso-HC 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 mouse endothelial cells (27) were cultured as described previously (9). Female SCCID (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 SCCID 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 was estimated by this analysis.

Cellular RIA. An indirect cellular RIA was used to determine the reactivities of anti-EDG mAbs with mouse 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)2 of affinity-purified goat anti-mouse 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 antiendothelial mAbs, i.e., mouse anti-EDG mAb (SN6h), mouse anti-human CD31 mAb, rat antimouse EDG (CD105) mAb, and rat antimonocese. In the immunostaining, isotype-matched control murine IgG (MOPC195 variant; IgG1-κ) and isotype-matched control rat IgG (R35–95; IgG2a-κ) were included as controls. For immunostaining of mouse tissues using mouse mAbs, InnoGenex Mouse-on-Mouse Iso-HC 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. Countersurrounding 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 skin grafts were stained with a mixture of SN6h and anti-human EDG-TARGETED ANTIANGIOGENIC THERAPY
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-expressing KM-3 leukemia cells and subconfluent proliferating HUVECs were carried out (Fig. 2). The results show equilibrium constants of $8.02 \times 10^9$, $2.85 \times 10^9$, and $1.01 \times 10^9$ liters/mol, respectively, for SN6f, SN6j, and SN6k to KM-3 cells. The equilibrium constants to HUVECs are $3.32 \times 10^9$, $3.08 \times 10^9$, and $1.01 \times 10^9$, respectively, for SN6f, SN6j, and SN6k. The results show that these mAbs bind to KM-3 cells and HUVECs with very similar avidities. The three mAbs all show good binding avidities, and the rank order of these mAbs for antibody avidity is SN6f > SN6j > SN6k. In the same analyses of Scatchard plot, the average number of antibody molecules bound per KM-3 cell was estimated to be $1.25 \times 10^4$, $1.75 \times 10^3$, and $1.70 \times 10^3$, respectively, at antibody saturation. The number per HUVEC was estimated to be $1.56 \times 10^9$, $1.13 \times 10^9$, and $1.52 \times 10^9$, respectively, for SN6f, SN6j, and SN6k. Therefore, the results show that these mAbs (all IgG1) are bivalent antibodies, the average number of antigens on these cell specimens is likely to be 1- to 2-fold greater than the antibody number. The present results show that EDG is highly expressed on proliferating HUVECs, and they express ~100-fold as many EDG molecules as do KM-3 cells.

Reactivities of Anti-EDG mAbs with Murine Endothelial Cells. An indirect cellular RIA was used to determine the reactivities of SN6f, SN6j, and SN6k with proliferating (subconfluent) SVEC4–10 murine endothelial cells by incubation for 2 or 24 h. The radioactivities (cpm) in the cells treated with SN6f, SN6j, SN6k, and an isotype-matched control IgG (IgG1-κ) for 2 h were $414 \pm 46$ (SD), $381 \pm 15$, $394 \pm 36$, and $290 \pm 29$, respectively. The radioactivities in the cells treated with the mAbs and control IgG for 24 h were $926 \pm 32$, $758 \pm 64$, $662 \pm 30$, and $456 \pm 60$, respectively. In the same assay, the radioactivities in proliferating HUVECs treated with SN6f and control IgG for 2 h were $8227 \pm 385$ and $196 \pm 24$, respectively. The results show that the binding of the three anti-EDG mAbs to SVEC4–10 murine endothelial cells is weak but significant compared with the binding of isotype-matched control IgG. The rank order of the three mAbs for reactivity with SVEC4–10 cells was SN6f > SN6j ≈ SN6k. The reactivities of these mAbs with HUVECs are much stronger than those with SVEC4–10 murine endothelial cells (see above and Ref. 15).

Suppression of Established Tumors by Systemic Administration of Naked mAbs. MCF-7 human breast cancer cells (8 10^6 cells/mouse) were injected i.d. into human skins that had been grafted to SCID mice, and the mice were left untreated until distinct palpable tumors appeared. Mice with distinct tumors were divided into groups ($n = 6$ or 5) and were treated by i.v. administration of PBS, an isotype-matched control IgG (MOPC 195 variant; IgG1-κ), SN6f, SN6j, SN6k, or SN6f plus SN6k (see “Materials and Methods” for details). SN6f and SN6k define mutually distant epitopes (15). The results are presented in Fig. 3. Both SN6j and SN6k showed significant antitumor efficacy, whereas SN6f was less effective. The results indicate that the antitumor efficacy of anti-EDG mAbs is not directly proportional to antigen-binding avidities of mAbs because SN6f shows the strongest antigen-binding avidity among the three mAbs to both HUVECs and murine endothelial cells (see above). The observed difference in the antitumor efficacy among SN6f, SN6j, and SN6k is consistent with the difference among the dgRA conjugates of the three mAbs (15). The results suggest the importance of other factors such as epitopes in the in vivo antitumor efficacy (see “Discussion”). The combination of SN6f and SN6k showed an additive effect. In additional tests, the effect of the mAb dose on the antitumor efficacy was investigated. An example of such tests is presented in Fig. 4. A 2-fold increase of SN6k from 200 μg to 400 μg resulted in a small increase in the antitumor efficacy. Both 200 μg and 400 μg of SN6k were

Statistical analysis of the data for the comparison of different groups of mice was carried out using Student’s t test.
Fig. 1. Immunostaining of blood vessels in the human skin/SCID mouse chimeras using species-specific anti-EDG (CD105) and anti-CD31 mAbs. Serial sections were cut from frozen tissues containing the human-mouse skins junction region (A–D; ×100). Tissue sections were immunostained with SN6h [an anti-human CD105 mAb (A)], anti-human CD31 mAb (B), anti-mouse CD105 mAb (C) and anti-mouse CD31 mAb (D). m, mouse skin; h, human skin. Mouse skin can be distinguished from human skin by the thin epidermis and hair follicles of the mouse skin. SN6h did not react with any blood vessels in either human or mouse skin (A). Antihuman CD31 mAbs stained numerous vessels in the human skin but not in the adjacent murine skin; arrows, a few examples of the stained human vessels (B). Antimouse CD105 mAbs (C) and antimouse CD31 mAbs (D) stained numerous vessels in both human skin and adjacent murine skin; arrowheads, examples of the stained mouse vessels. In an additional study, serial sections were cut from frozen tissues of the grafted human skins bearing large (250 mm³) MCF-7 human tumors (E and F; ×200). SN6h stained multiple vessels in the tumor in the human skin but not in the adjacent murine s.c. tissue (E). Antimouse CD105 mAbs stained multiple vessels in the tumor in the human skin and the murine s.c. tissue (F). Arrows (E), several examples of stained human vessels; arrowheads (F), several examples of stained mouse vessels. Isotype-matched murine control IgG (IgG1-κ) and isotype-matched rat control IgG (IgG2a-κ) showed no significant staining. The staining patterns were similar to that in A.

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

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tumors were distributed into four groups (n = 8). 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 mm³, 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 ¹²⁵I-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 µg), a mAb (200 µg), or a mixture of SN6f (200 µg) and SN6k (200 µg) 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 µg), 200 µg SN6k, or 400 µg 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 µg of an isotype-matched control IgG, 200 µg SN6j, or 400 µg 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 chimera 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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**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. *a*, 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.

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

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<tr>
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<td>0.739</td>
<td>0.549</td>
<td>0.426</td>
<td>1.289</td>
</tr>
</tbody>
</table>

*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.  
‡ (Mean FTV of SN6j) × (mean FTV of CPA).  
§ 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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should be noted that injections of PBS in the lower line and CPA in the upper line resulted in similar size tumors in individual SCID mice. Consequently, the average size of tumors in each group became similar at the onset of therapy (see Fig. 6). Arrowheads, 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.

“Introduction”). In contrast, no toxicity was detected for naked anti-EDG mAb SN6j in a dose-escalation study; the maximum tolerated dose was more than 0.344 mg/g body weight of mice.5

Previously, Yan et al. (22) developed human skin/SCID mouse chimeras and immunostained blood vessels in a human skin graft and adjacent murine skin using antimouse PECAM (CD31) mAb, anti-human CD31 mAb, and 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 antimouse CD105 mAb and SN6h, an antihuman 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 antimouse CD105 mAb and antihuman 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 differences 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 SN6kdgRA 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.
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.

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 sprouts 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 immuno-competent 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 desirable 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 tumorogenesis. Recently, Ananth et al. (54) reported that the neutralizing antibody against TGF-β inhibited tumorogenesis 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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REFERENCES

1. Haruta, Y., and Seon, B. K. Distinct human leukemia-associated cell surface glyco-


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

Norihiko Takahashi, Akinao Haba, Fumihiko Matsuno, et al.


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