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Anti-endoglin monoclonal antibodies are effective for suppressing metastasis and the primary tumors by targeting tumor vasculature

Shima Uneda, Hirofumi Toi, Tomoko Tsujie, Masanori Tsujie, Naoko Harada, Hilda Tsai, and Ben K. Seon*

Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY

Abstract

Anti-metastatic activity of an antitumor agent is exceedingly important because metastasis is the primary cause of death for most solid cancer patients. In this report, we show that 3 anti-endoglin (ENG) monoclonal antibodies (mAbs) SN6a, SN6j and SN6k which define individually distinct epitopes of ENG of tumor vasculature are capable of suppressing tumor metastases in the multiple metastasis models. The metastasis models were generated by i.v., s.c. (into flank) or mammary gland fat pad injection of 4T1 murine mammary carcinoma cells and splenic injection of two types of colon26 murine colorectal carcinoma cells. Individual mAbs were injected i.v. via the tail vein of mice. SN6a and SN6j effectively suppressed the formation of metastatic colonies of 4T1 in the lung in all of the three 4T1 metastatic models. In addition, these mAbs were effective for suppressing the primary tumors of 4T1 in the skin and mammary fat pad. These mAbs effectively suppressed microvessel density and angiogenesis in tumors as measured by the Matrigel plug assay in mice. No significant side effects of the administered mAbs were detected. Furthermore, SN6a and SN6j extended survival of the tumor-bearing mice. SN6j, SN6k and their immunoconjugates with deglycosylated ricin A-chain were all effective for suppressing hepatic metastasis of colon26. The findings in the present study are clinically relevant in view of the ongoing clinical trial of a humanized (chimerized) form of SN6j.

Keywords

endoglin; metastasis; monoclonal antibodies; antiangiogenesis; vascular targeting

Metastasis is the primary cause of death for most cancer patients.^{1–4} Approximately 90% of cancer deaths result from the metastatic spread of the primary solid tumors. Therefore, development of a new anticancer agent that is effective against tumor metastasis will be extremely important. In the present study, we investigated anti-metastatic activity of three anti-ENG mAbs that define individually distinct epitopes of ENG.

A homodimeric cell surface glycoprotein, later termed ENG (CD105), was initially identified as a human leukemia-associated cell membrane antigen.^{5,6} ENG (CD105) is mainly expressed on immature B-lineage/myeloid leukemia cells and endothelial cells.⁵⁻⁷ Two isoforms of ENG, L-ENG and S-ENG, differing in the size of their cytoplasmic tails, have been characterized.⁸ ENG is a proliferation-associated cell membrane antigen⁹⁻¹² and strongly expressed on the tumor-associated angiogenic vascular endothelium.^{10,11,13} In addition, ENG is essential for angiogenesis^{14,15} and a component of the transforming growth factor (TGF)- β receptor complex.¹⁶ Yamashita *et al*¹⁷ and others¹⁸ reported that ENG forms a heterodimeric complex with TGF- β receptors I and II. L-ENG and S-ENG may differentially modulate TGF- β signaling.¹⁹ Recently, several studies indicated that ENG represents a more specific and sensitive marker for tumor angiogenesis and/or tumor progression than the commonly used pan-endothelial markers such as CD34 and CD31 in various types of human malignancies.²⁰⁻²³

Previously, we showed that immunoconjugates (immunotoxins and radioimmunoconjugates) and naked form of selected anti-ENG mAbs were effective for tumor suppression by targeting angiogenic vasculature in mice.^{11,24-28} In these studies, we targeted primary tumors grown in SCID mice,^{11,24,25} immunocompetent mice^{27,28} and human skin/SCID mouse chimeras in which human tumors were implanted in human skins grafted into SCID mice.²⁶ These studies were performed by targeting ENG of tumor vasculature in subcutaneous tumors. To facilitate clinical application, we generated a humanized (chimerized) form of an anti-ENG mAb, termed c-SN6j, and performed studies of pharmacokinetics, toxicology and immunogenicity of c-SN6j in nonhuman primates.²⁹ No significant toxicity was detected by several criteria and minimal immune response to the murine part of c-SN6j was detected by multiple i.v. injections. The results support our hypothesis that c-SN6j can be safely administered in cancer patients. Recently, we showed that immune status of the hosts plays an important regulatory role in the ENG-targeted vascular targeting therapy.²⁸

In the present study, we investigated anti-metastasis activities of three anti-ENG mAbs (SN6a, SN6j and SN6k) in multiple metastasis models including mammary carcinoma and colorectal carcinoma. These mAbs showed significant anti-metastatic activities in all of the different metastasis models. We anticipated that our anti-ENG mAbs will be effective for suppressing metastasis irrespective of tumor types because these mAbs target tumor vasculature but not tumor cells *per se*.

These mAbs do not react with either 4T1 or colon26 murine tumor cells^{27,28} but weakly cross-react with mouse endothelial cells^{24,28} and suppress angiogenesis in mice.^{24,28} The above hypothesis/anticipation was supported by the present test results. The results in this report will be clinically relevant because a multicenter phase I clinical trial of c-SN6j (also known as TRC105) is in progress.

Material and methods

Cells and animals

4T1 murine mammary carcinoma cell line was obtained from American Type Culture Collection (Rockville, MD). The parental clone L0 and a subclone L5 of colon26 murine colon adenocarcinoma cell line were kindly provided by Drs. Norihiko Takahashi and Masao Kondo (Hokkaido University Medical School, Hokkaido, Japan). Both 4T1 and colon26 were cultured in a monolayer in RPMI 1640 media containing 10% fetal bovine serum (FBS), 100 unit/ml penicillin, 100 µg/ml streptomycin and 250 ng/ml amphotericin B in a humidified atmosphere with 5% CO₂ at 37°C. Human leukemia cell lines KM-3 and MOLT-4 that were used in a competitive binding assay^{30,31} to compare epitopes defined by different anti-ENG mAbs (see Results) were cultured as describe previously.⁵ Six-weeks-old female BALB/c mice were obtained from the National Cancer Institute. Mice were housed in a protected environment in a laminar flow unit and given sterilized food and water on a 12-hr light/dark cycle. All handling of the mice was performed in a laminar flow hood.

mAbs and reagents

Anti-human ENG mAbs SN6j, SN6k and SN6a, that show weak cross-reactivity with mouse endothelial cells, were generated in our laboratory and reported previously.^{24,30} The 2 control IgGs, MOPC195 variant (MOPC195v; IgG1-κ) and RPC5 (IgG2a-κ) were purified from corresponding ascites samples in our laboratory. Immunotoxins were prepared by conjugating deglycosylated ricin A chain (dgRA) to SN6j, SN6k or an isotype-matched control IgG (MOPC195v) as described previously.¹¹ Mouse serum albumin was obtained from Sigma (St. Louis, MO). Antibodies were centrifuged at 100,000g at 4°C for 1 hr, 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 antibodies were diluted with sterile PBS containing mouse serum albumin (0.05% final concentration).

Matrigel plug assay in mice

This assay was performed as described previously.²⁸ Briefly, 0.375 ml of Matrigel Matrix (BD Biosciences, San Diego, CA) was mixed with 1.0×10^5 4T1 cells in 0.125 ml culture media and implanted into the left flank of mice. One day after the implantation, mice were divided into 2 groups (7 mice per group) by evenly distributing mice with similar BW and plug size. The mice were treated by injections of 1.8 µg/g BW of an anti-ENG mAb or isotype-matched control IgG *via* tail vein on day 1, 4 and 7. Ten days after the implantation, Matrigel plugs were removed and fixed in zinc fixative (BD Biosciences) for 24 hr at room temperature, and stained with anti-mouse CD31 mAb using LSAB+ system-HRP (horse radish peroxidase) from Dako (Carpinteria, CA) according to manufacturer's instruction with minor modifications.²⁸ For the quantification of microvessel density (MVD), 12 hotspot fields (4 fields × 3 samples) of CD31 staining at ×200 were captured from each group using Spot digital camera (Diagnostic Instruments, Sterling Heights, MI) mounted to Nikon ECLIPSE E600 (Kawasaki, Japan).²⁶

Cell preparation for transplantation into mice

Cultured 4T1 and colon26 cells were harvested using Hanks solution containing 3-mM EDTA and 25-mM HEPES, washed twice and then re-suspended in PBS, pH 7.2. Cells suspension was inoculated using a 30G1/2 needle (BD 30G1/2 PrecisionGlide Needle; Becton Dickinson, Franklin Lakes, NJ) to establish each tumor model.

Transplantation of 4T1

4T1 cells were inoculated into mammary gland fat pad, s.c. tissues of flank, or tail vein of mice to generate 3 different tumor models of 4T1. To this end, 1.0×10^5 cells in 0.1 ml PBS were inoculated into the left mammary gland fat pad or left flank of individual mice of two separate groups while 2.0×10^4 cells in 0.2 ml PBS were injected into tail vein of individual mice of another group. Preliminary titration experiments showed that under these 3 conditions, 4T1 effectively formed metastasis colonies in the lung.

Transplantation of colon26 cells into spleen of the mice

To generate a hepatic metastasis model of colon26, mice were anesthetized with ketamin / midazolam by i.p. injection. After small incision was inflicted at upper midline of abdomen, spleen was carefully exposed and 2×10^4 colon26 cells (in 0.1 ml of PBS) were injected slowly under capsule of the spleen. After verification of hemostasis, the spleen was returned into the peritoneal cavity and then the abdominal wall was sutured with 4-0 VICRYL suture (Ethicon, Somerville, NJ).

Therapy of mice bearing 4T1 and colon26 tumors

In all therapeutic studies, mAb or control IgG was injected i.v. *via* the tail vein of individual mice. For the group of mice who received 4T1 cells s.c. into the flank, mice bearing established s.c. tumors were selected and treated by i.v. administration of a mAb (1.8 μ g/g BW) or an isotype-matched control IgG (1.8 μ g/g BW). The therapy was initiated 3 days after the tumor inoculation and the treatment was repeated on days 6, 9 and 16. For the group of mice who received 4T1 cells into mammary gland fat pad, two sets of therapeutic experiments were performed. One set of the mice was treated as described above while another set of mice was treated by 6 injections of a mAb or control IgG at 3-day intervals. For the group of mice who received i.v. inoculation of 4T1 cells, therapy was initiated by i.v. administration of a mAb (1.8 μ g/g BW) or control IgG (1.8 μ g/g BW) 1 day after tumor inoculation and the treatment was repeated 3 times at 3 day intervals. Mice were sacrificed at slightly different times for different experiments, for example, on day 27 (see the text for the details).

For the groups of mice who received splenic inoculation of colon26 cells, therapy was initiated by i.v. administration of a control (PBS), mAb (17 μ g/mouse), control IgG (17 μ g/mouse), immunotoxin (20 μ g/mouse) or control immunotoxin (20 μ g/mouse) 2 days after tumor inoculation. We used 2 clones of colon26. One is the parental clone L0³² and the other is a highly metastatic subclone L5.³³ The treatment of the colon26 inoculated mice was repeated twice (for L0) or 3 times (for L5) at 2 days intervals. Mice were sacrificed on day 15 and liver was excised to count the metastasis colonies.

Follow-up of treatment efficacy

During the therapy, mice were monitored daily for morbidity. Size of 4T1 s.c. tumors and body weight (BW) of the mice were monitored every other day. Size of the tumor was measured using an electronic digital caliper (Pro-Max, Fread V. Foulmer Company, Newton, MA) that was connected to a computer using Gage-Wedge software (Fowler). The measured tumor diameters were converted to tumor volumes using Excel software (Microsoft, Redmond, WA) and the following formula: $V = \text{length} \times \text{width} \times \text{height} \times \pi/6$. Mice and weight of organs were measured using an electronic balance (Scout Pro 202, Ohaus Corp., Pine Brook, NJ). After the mice bearing tumors were sacrificed at the end of experiments, lung (for 4T1) or liver (for colon26) was excised to count metastasis colonies. In addition, the primary tumors were excised to measure the size and weight of the excised tumors.

Analysis of number of the metastatic tumors

The mice who received splenic inoculation of colon26 were sacrificed 15 days after tumor inoculation to count the metastatic colonies in the liver. The mice who received 4T1 inoculation into mammary gland fat pad and s.c. tissues of the left flank were sacrificed on day 24 and 27, respectively while the mice who received 4T1 i.v. were sacrificed on day 16. Liver and spleen from the colon26 tumor-bearing mice were weighed while lung from the 4T1 tumor-bearing mice was weighed. Metastatic tumor colonies were counted under a stereoscopic microscope (SMZ800, Nikon, Tokyo, Japan) after the tissues were fixed with Bouin's solution (Ricca Chemical, Arlington, TX).

Statistical analysis and assessment

Statistical analysis of the data was performed using the Mann-Whitney *U* test and the Student's *t*-test. Survival of mice was evaluated by Kaplan-Meier analysis and Wilcoxon's test (StatView software, version 5, Abacus concepts, Berkeley, CA).

Results

Comparison between epitopes defined by different anti-ENG mAbs

We performed a competitive binding assay to gain information about the spatial relationship between epitopes defined by SN6a, SN6j, SN6k and SN6, the prototype anti-ENG mAb.⁵ The assay was performed as described previously.³¹ The test results show that each of the epitopes defined by SN6, SN6a, SN6j and SN6k is at mutually separate locations (data not shown).

Effect of anti-ENG mAb on angiogenic blood vessels in mice

Matrigel plug assay was performed to investigate the effect of anti-ENG mAb on angiogenesis in mice. A mixture of Matrigel Matrix and 4T1 cells was implanted s.c. into the left flank of individual mice. Matrigel matrix facilitates angiogenesis. The mice were evenly divided into 2 groups (see Materials and Methods for details) and individual groups of mice were treated by i.v. injections of an anti-ENG mAb or isotype-matched control IgG. Matrigel plugs were removed 10 days after the implantation, and microvessel density (MVD) was assessed by immunostaining with anti-mouse CD31 mAb. Representative

images are shown in Figure 1a. MVD of Matrigel plugs of SN6a-treated mice was significantly lower than that of an isotype-matched control IgG-treated mice ($p < 0.01$; Fig. 1b). Moreover, the vessels in plugs of SN6a-treated mice showed shrinkage and attenuation when compared with those of the control IgG-treated mice. SN6j showed a similar antiangiogenic effect on MVD in colon26 tumor in the Matrigel plug assay.²⁸

Suppression of the s.c. tumors and metastasis of 4T1

4T1 that is inoculated s.c. metastasizes to the lung.³⁴ In the present set of experiments, 4T1 was inoculated s.c. into the left flank of mice. Mice with established tumors were distributed evenly (based on the tumor size) into 2 groups. Then, SN6j or an isotype-matched control IgG (MOPC195v; IgG1- κ) was administered i.v. into each group of mice ($n = 22$ for each group). The results are shown in Figure 2. SN6j showed significant suppressive activity against the established s.c. tumors. Difference in the tumor size between the SN6j-treated group and the isotypematched control IgG (MOPC195v)-treated group is statistically significant ($p < 0.05$) after day 15 and at the end of experiment, that is, day 24. Average tumor sizes of the MOPC195v- and the SN6j-treated group are $201.9 \pm 74.6 \text{ mm}^3$ and $152.2 \pm 55.4 \text{ mm}^3$, respectively, on day 15, $508.5 \pm 254.1 \text{ mm}^3$ and $370.0 \pm 151.0 \text{ mm}^3$, respectively, on day 21 and $888.8 \pm 427.5 \text{ mm}^3$ and $662.6 \pm 285.8 \text{ mm}^3$, respectively, on day 24 (Fig. 2a). Tumor sizes of individual mice that were treated with MOPC195v and SN6j are shown in Figures 2b and 2c, respectively. Growth pattern of the tumors is more homogeneous in the SN6j-treated mice than in the MOPC195v-treated mice. A representative example of the mice bearing the s.c. tumors is illustrated for the MOPC195v-treated group and the SN6j-treated group (Fig. 2d). Mice were sacrificed on day 24 and the number of metastasis colonies in the lung was counted. The number of the metastasis colonies was 20.8 ± 16.4 and 10.4 ± 11.2 , respectively, for the MOPC195v-treated ($n = 22$) and SN6j-treated mice ($n = 22$) (Fig. 2e). Difference in the colony number between the two groups of mice was statistically significant ($p = 0.015$). The results demonstrate that SN6j is effective for suppressing tumor metastasis as well as suppressing growth of the primary tumors. Growth pattern of the body weights of individual mice receiving SN6j were similar to that of mice receiving the control IgG during therapy and no significant side effects of the administered SN6j were detected.

Suppression of 4T1 tumors in mammary glands and lung

4T1 was inoculated into the mammary gland fat pad of mice. Mice bearing palpable tumors were evenly distributed into 4 groups 3 days after the tumor inoculation. Each group of the mice was treated by i.v. administration of SN6j, an isotype-matched control IgG (MOPC195v; IgG1- κ), SN6a or an isotype-matched control IgG (RPC5; IgG2a- κ). Tumor growth patterns in the individual mice of the 4 groups are shown in Figures 3a'–3e'. The average tumor growth pattern for each group is shown in Figure 3a. Difference in the tumor size between the control MOPC195v-treated group and the SN6j-treated group is statistically significant ($p < 0.03$) after day 13 and at the end of experiment, that is, day 27. The average tumor volumes of the MOPC195v- and SN6j-treated group are $92.7 \pm 23.3 \text{ mm}^3$ and $65.5 \pm 29.3 \text{ mm}^3$, respectively, on day 15 ($p < 0.03$), $359.3 \pm 84.0 \text{ mm}^3$ and $292.3 \pm 92.0 \text{ mm}^3$, respectively, on day 23 ($p < 0.03$) and $689.4 \pm 162.2 \text{ mm}^3$ and $496.9 \pm 154.9 \text{ mm}^3$, respectively, on day 27 ($p < 0.01$). Similarly, difference in the tumor size between the

RPC5-treated group and SN6a-treated group is statistically significant ($p < 0.05$) after day 13 and at the end of the experiment, that is, day 27 (Fig. 3a'). The average tumor volume of the RPC5- and SN6a-treated group was $85.3 \pm 26.5 \text{ mm}^3$ and $61.6 \pm 16.6 \text{ mm}^3$, respectively, on day 15 ($p < 0.05$), $377.0 \pm 92.1 \text{ mm}^3$ and $307.5 \pm 82.6 \text{ mm}^3$, respectively, on day 23 ($p < 0.01$) and $695.9 \pm 198.2 \text{ mm}^3$ and $543.6 \pm 110.2 \text{ mm}^3$, respectively, on day 27 ($p < 0.01$).

Accurate measurement of the tumor thickness is difficult for the tumors formed in mammary glands. Therefore, we performed an additional experiment to measure the tumor size by excising the tumors and measuring the size of the excised tumors after the mice were sacrificed at the end of the experiment, that is, on day 27 (Fig. 3b). Additionally lungs were excised from the sacrificed mice to measure the metastasis colonies in the lungs (Fig. 3c). The average size of the excised tumors was $760.5 \pm 227.6 \text{ mm}^3$ and $502.2 \pm 145.5 \text{ mm}^3$, respectively, for the MOPC195v-treated and SN6j-treated group. The difference is statistically significant ($p = 0.0002$, Fig. 3b). The size of excised tumors from the RPC5-treated and SN6a-treated group was $755.4 \pm 261.1 \text{ mm}^3$ and $537.7 \pm 151.3 \text{ mm}^3$, respectively, and the difference was statistically significant ($p < 0.003$). The results of excised tumors support our conclusion that was based on the direct measurement of tumors in the mice during therapy (Fig. 3a). Both results show that SN6j and SN6a are effective for suppressing growth of the primary tumors.

The average number of the metastatic colonies in the lung was 25.0 ± 21.9 and 10.6 ± 12.0 , respectively, for the MOPC195v-treated ($n = 20$) and SN6j-treated group ($n = 19$) (Fig. 3c). The difference is statistically significant ($p < 0.01$). The average number of tumor colonies in the lung was 21.5 ± 13.9 and 10.3 ± 7.6 , respectively, for the RPC5-treated ($n = 19$) and SN6a-treated group ($n = 20$) (Fig. 3c). The difference is statistically significant ($p < 0.01$). The results show that both SN6j and SN6a are effective for suppressing metastasis as well as the primary tumors.

Prolongation of survival of mice bearing 4T1 tumors

We investigated the effect of SN6j and SN6a on the survival of the 4T1 tumor-bearing mice. To this end, 4T1 (1.0×10^5 cells) was inoculated into the mammary gland fat pad. Mice bearing palpable tumors were evenly (based on tumor size) distributed into 4 groups 3 days after tumor inoculation. Groups of mice were treated with i.v. administration of $1.8 \mu\text{g/g BW}$ of SN6j ($n = 19$), an isotype-matched control IgG (MOPC195v; $n = 19$), SN6a ($n = 18$) or an isotype-matched control IgG (RPC5; $n = 18$) on day 3, 6, 9 and 16. Survival of the mice was followed during the therapy (Fig. 4b). To investigate the correlation of a decrease in the lung metastasis with prolonged survival of the tumor-bearing mice, 10 mice (for each of the SN6j and MOPC195v group) or 9 mice (for each of the SN6a and RPC5 group) from different groups were sacrificed on day 24 and metastasis colonies in the lungs from individual mice were counted (Fig. 4a). Treatment of the tumorbearing mice with either SN6j or SN6a decreased metastasis when compared with the respective control groups which is consistent with the earlier results of another set of experiments (Fig. 3c). Survival of the remaining mice ($n = 9$ for each group) was followed until day 32 (Fig. 4b). The results show that treatment with either SN6j or SN6a prolonged survival of the tumor-bearing mice ($p < 0.03$).

and $p < 0.02$, respectively). Furthermore, the results show the correlation of a decrease in the metastatic colonies in the lung with the prolonged survival of tumor-bearing mice.

Antitumor efficacy of SN6j and SN6a against intravenously injected 4T1

We investigated the effect of SN6j and SN6a on the tumor colonies in the lung after mice received 4T1 i.v. via tail vein. The tumor inoculated mice were divided into 3 groups ($n = 10$ for each group) and treated with SN6j, SN6a or control IgG (RPC5) on day 3, 6, 9 and 12 after tumor inoculation. Mice were sacrificed on day 16 and number of tumor colonies in the lung as well as weight of the lung was determined. Number of tumor colonies in the lung was 80.6 ± 30.1 , 31.8 ± 21.7 and 30.2 ± 14.3 , respectively, for the RPC5-treated, SN6j-treated and SN6a-treated group. The difference in the number between the SN6j-treated/SN6a-treated groups and the control group is statistically significant ($p < 0.01$ for both SN6j vs. control and SN6a vs. control) (Fig. 5a). Weights of the lungs from the mice treated with the control IgG (RPC5)-treated, SN6j-treated and SN6a-treated group were 0.202 ± 0.031 , 0.162 ± 0.036 and 0.168 ± 0.024 g. Thus, the weights of the lungs of the mice treated with anti-ENG mAbs are significantly lower than that of the control mice ($p < 0.003$ between RPC5 and SN6j; $p = 0.02$ between RPC5 and SN6a) (Fig. 5b). The results show that SN6j and SN6a are effective for suppressing tumor formation in the lung by circulating 4T1 tumor cells.

Suppression of hepatic metastasis of colon26 colon carcinoma

Our anti-ENG mAbs that target ENG on tumor vasculature but not on tumor cells are expected to be effective for suppressing tumor metastasis irrespective of tumor types. These mAbs do not react with either 4T1 or colon26^{27,28} but weakly cross-react with mouse endothelial cells^{24,28} and suppress angiogenesis in mice.^{24,28} To test the above hypothesis of the wide anti-metastatic activity of anti-ENG mAbs, we investigated anti-metastatic activities of 3 anti-ENG mAbs SN6a, SN6j and SN6k against hepatic metastasis of colon26 colon carcinoma in addition to the metastases of mammary carcinoma. We used 2 types of colon26; 1 is the parental clone L0 (Figs. 6a and 6b) while the other is a highly metastatic subclone L5 (Figs. 6c and 6d).

Test results of the L0 inoculated mice are shown in Figure 6a. Mice inoculated with L0 were divided into 5 groups on day 2 and treated with control (PBS), SN6k (17 μ g/mouse), SN6j (17 μ g/mouse), control conjugate (MOPC-dgRA; 20 μ g/mouse) or SN6k-dgRA (20 μ g/mouse). The conjugates were prepared by coupling a mAb or an isotype-matched control IgG to the deglycosylated A chain subunit of ricin (dgRA) as described previously.¹¹ The therapy was repeated twice at 2 day intervals, that is, on day 4 and 6, and mice were sacrificed on day 15 to count metastatic colonies in the liver. The average numbers of metastatic colonies in the livers of groups of mice treated with control (PBS), SN6k, SN6j, control conjugate (MOPC-dgRA) or SN6k-dgRA were 10.1 ± 7.2 , 3.1 ± 1.7 , 3.2 ± 1.4 , 7.5 ± 4.8 and 2.1 ± 1.8 , respectively (Fig. 6a). Numbers of the metastatic colonies of the SN6k-, SN6j- and SN6k-dgRA-treated groups were significantly lower than that of the control group ($p < 0.03$, < 0.03 and $= 0.01$, respectively). The number of the SN6k-dgRA-treated group is also significantly less than that of the MOPC-dgRA-treated group ($p < 0.05$). Thus, SN6k, SN6j and SN6k-dgRA were all effective for suppressing hepatic metastasis of L0 (Fig. 6a).

Examples of livers from the SN6k-dgRA-treated mice and control mice are shown in Figure 6b.

In another set of experiments, a highly metastatic subclone L5 of colon26³³ was inoculated into spleens of mice and the mice were divided into 4 groups on day 2. Groups of mice were treated with control, SN6j, MOPC-dgRA or SN6j-dgRA as described above but the therapy was repeated 3 times on day 4, 6 and 8 in view of the stronger metastatic capacity of L5 when compared with L0. The average numbers of the metastatic colonies for the control, the SN6j-treated, MOPC-dgRA-treated and SN6j-dgRA-treated groups were 48.1 ± 43.2 , 9.4 ± 9.1 , 19.6 ± 17.1 and 7.5 ± 10.6 , respectively (Fig. 6c). Treatment with SN6j and SN6j-dgRA significantly decreased the number of tumor colonies when compared with the control group ($p < 0.03$ and < 0.02 , respectively). The average metastatic colony number (7.5 ± 10.6) of the SN6j-dgRA-treated group is substantially lower than that (19.6 ± 17.1) of the MOPC-dgRA-treated group. Nevertheless, the difference showed a trend to the statistical significance ($p = 0.12$).

In the third set of the colon26 experiments, mice inoculated with L5 were treated with SN6a, control IgG (RPC5) or PBS (control). The results are shown in Figure 6d. The average metastatic colonies for the control group, RPC5-treated group and SN6a-treated group were 46.3 ± 41.6 , 50.43 ± 25.23 and 3.9 ± 26.3 . The suppressive activity of SN6a against the hepatic metastasis of L5 is statistically significant when compared with the control ($p = 0.02$) and the control IgG ($p = 0.02$).

Differences in the metastasis colony number between mice treated with naked anti-ENG mAbs (SN6k and SN6j) and those treated with their immunoconjugates are small and statistically insignificant. Potential reasons for these relatively small differences are discussed below in the Discussion.

Discussion

Antiangiogenic therapy of cancer is a highly attractive new approach to cancer therapy.³⁵ It can potentially overcome major problems associated with other therapeutic agents of solid cancers, that is, the problems of poor delivery^{36,37} and tumor heterogeneity. In addition, 1 antiangiogenic agent may be applied to therapy of many different cancers. However, most, if not all, of the currently approved antiangiogenic drugs either block VEGF or VEGF receptors.³⁸ Therefore, it will be important to develop other types of antiangiogenic agents for cancer therapy. Furthermore, vascular targeting antiangiogenic therapy (VT-AAT) may present an additional advantage over conventional AAT in that it may be able to better attack/destroy the pre-existing blood vessels of established tumors in addition to inhibition of neovasculature in conventional AAT.³⁹⁻⁴² However, the major obstacle in VT-AAT is the difficulty in finding an appropriate target that allows us to selectively attack tumor vasculature without causing major damages of normal vital organs. We hypothesized that ENG can be such a target. First, expression of ENG is relatively restricted.^{5,7,43} Second, ENG is a proliferation-associated antigen on endothelial cells and its expression is up-regulated in tumor-associated vascular endothelium.⁹⁻¹³ Third, turnover of endothelial cells of normal adult tissue vasculature is very slow (*e.g.*, more than 1,000 days) while these

endothelial cells undergo rapid proliferation during spurts of angiogenesis in tumors.^{39,44} Therefore, the rapidly dividing endothelial cells of tumor vasculature will be much more susceptible to killing by anti-ENG agents than the quiescent vascular endothelium of normal tissues. Among the potential anti-VT-AAT agents, we chose mAbs that can be highly specific for a particular target if appropriate mAbs are generated and selected. We have previously developed a unique system to generate highly specific mAbs targeting cell membrane antigens.^{5,45,46} In fact we have previously identified ENG using 1 of these mAbs.⁵ To test our hypothesis that ENG will be a useful target for tumor vasculature in VT-AAT, we performed several *in vitro* and animal model studies; these animal models include SCID mice bearing human tumors,^{11,24,25} human skin/SCID mouse chimeras bearing human tumors,²⁶ immunocompetent mice bearing syngenic tumors^{27,28} and nonhuman primates.²⁹ These studies showed that anti-ENG immunoconjugates (immunotoxins and radioimmunoconjugates) and naked anti-ENG mAbs can selectively target tumor vasculature in the primary tumors without causing major side effects in animals.

Several mechanisms may contribute to the antiangiogenesis/antitumor activity of these anti-ENG mAbs.²⁸ For instance, certain anti-ENG mAbs exert direct suppression of endothelial cell proliferation *in vitro* in the absence of any accessory cells³¹ and induce apoptosis.²⁸ In addition, CD4⁺ and CD8⁺ T cells play major roles in the *in vivo* antitumor efficacy of anti-ENG mAb SN6j.²⁸ Furthermore, SN6j shows strong ADCC (unpublished). Other potential mechanisms including activation of signaling pathways by anti-ENG mAbs in endothelial cells are under study.

On the other hand, Burrows *et al.*¹⁰ and Munoz *et al.*⁴⁷ reported that their anti-ENG immunotoxins could kill ENG-expressing cells *in vitro* while Volkel *et al.*⁴⁸ reported anti-ENG immunoliposomes that showed rapid and strong binding to ENG-expressing cells. Other investigators reported selective localization of radiolabeled anti-ENG mAbs in a canine mammary carcinoma model,⁴⁹ mice bearing B16 melanoma⁵⁰ and excised kidneys from renal carcinoma patients.⁵¹

In spite of these and other interesting reports, the potential anti-metastasis activity of anti-ENG mAbs was not previously reported. In view of the importance of metastasis in cancer therapy, we studied anti-metastasis activities of anti-ENG mAbs and report the results here. In this study, we used 3 anti-ENG mAbs (*i.e.*, SN6a, SN6j and SN6k) and 5 metastasis animal models including mammary carcinoma (3 animal models) and colon carcinoma (2 animal models). These mAbs showed substantial anti-metastasis activities, and metastasis was suppressed by anti-ENG mAbs in each of the 5 metastasis models. In addition, growth of the primary tumors was also suppressed by the anti-ENG mAbs in the animal models when growth of the primary tumors was followed during the therapy. SN6a effectively decreased MVD in 4T1 tumor and induced shrinkage or attenuation of the remaining tumor blood vessels in the Matrigel plug assay (Fig. 1) as SN6j did against MVD in colon26 tumor.²⁸ Body weights of individual mice receiving a mAb gradually increased during the therapy as those of mice receiving a control IgG and no significant side effects of the administered mAbs were detected. The differences in the metastatic colony number between mice treated with naked mAbs (SN6k and SN6j) and those treated with their immunoconjugates (SN6k-dgRA and SN6j-dgRA) are relatively small (Fig. 6). We postulate

that these small differences may be a consequence of the relatively strong antitumor efficacy of naked anti-ENG mAbs against metastatic tumors. This postulation appears to be consistent with the results of an ongoing clinical trial of a naked form of anti-ENG mAb in cancer patients with metastatic/advanced diseases (see below).

A next critical question would be the relevance of the reported animal and *in vitro* studies to cancer therapy in patients. In this regard, recently we initiated a multicenter phase I clinical trial of a humanized (chimerized) form of SN6j (c-SN6j, TRC105) in patients with advanced and/or metastatic solid cancers for whom curative therapy is not available (ClinicalTrials.gov Identifier: NCT00582985). Although this clinical trial is still in progress, the early results of this trial are consistent with our hypothesis that an appropriately selected anti-ENG mAb can effectively target tumor vasculature without causing major side effects in cancer patients. Sustained efforts in both laboratory studies and clinical trials will be important for the successful application of anti-ENG mAbs and their derivatives (*e.g.*, immunoconjugates) to therapy of cancer and other angiogenesis-associated diseases. We intend to exert such efforts.

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Abbreviations

BW	body weight
dgRA	deglycosylated ricin A chain
ENG	endoglin
mAb	monoclonal antibody
MSA	mouse serum albumin
MVD	microvessel density
TGF	transforming growth factor
VT-AAT	vascular targeting antiangiogenic therapy

Figure 1.

Effect of anti-ENG mAb on angiogenesis in mice. Matrigel matrix mixed with 1.0×10^5 4T1 cells was injected s.c. in the left flank of BALB/c mice on day 0. The mice were treated with 1.8 $\mu\text{g/g}$ BW of SN6a or isotype-matched control IgG *via* tail vein on day 1, 4 and 7. The matrigel plugs were fixed on day 10 for immunohistochemical staining. (a) Examples of rat anti-mouse CD31 (PECAM-1) mAb staining of matrigel plug sections from SN6a-treated (panels a', c' and e') and control IgG-treated (b', d' and f') mice ($\times 200$). The sections were counterstained with hematoxylin. Arrowheads in the figure indicate representative CD31 positive vessels. (b) The CD31 positive vessels in hotspots were counted in 4 separate $\times 200$ fields from 5 samples per group.

Figure 2.

Suppression of the primary tumors and lung metastasis of the s.c. inoculated 4T1 mammary carcinoma cells. 4T1 (1.0×10^5 cells/mouse) was inoculated s.c. into the left flank of mice. (a) The average size of the primary s.c. tumors is shown for the mice treated with SN6j ($n = 22$) and the mice treated with an isotype-matched control IgG (MOPC195v; IgG1- κ) ($n = 22$). Difference in the tumor size between the 2 groups is statistically significant (*, $p < 0.01$). (b) Tumor sizes of individual mice treated with MOPC195v. (c) Tumor sizes of individual mice treated with SN6j. (d) Representative examples of the mice treated with either control IgG (left panel) or SN6j (right panel). (e) Number of metastasis colonies in the lung of the mice treated with control IgG or SN6j ($n = 22$ in each group). The difference is statistically significant ($p = 0.015$).

Figure 3.

Effect of the systemically administered SN6j and SN6a on the 4T1 lung metastasis and the primary tumors in the mammary glands. 4T1 (1.0×10^5 cells) was inoculated into mammary gland fat pad. (a) a', The average tumor size in each of the 4 groups of mice that were treated with SN6j, an isotype-matched control IgG (MOPC195v; IgG1- κ), SN6a or an isotype-matched control IgG (RPC5; IgG2a- κ). The vertical arrows indicate injections of a mAb or control IgG. Antitumor efficacy of SN6j and SN6a is statistically significant compared with that of the respective isotype-matched control IgG (see the text for details). (a) b'-e', tumor growth patterns in individual mice of the 4 groups. (b) Tumors in individual mice were excised at the end of the experiment, that is, on day 27, and the size of individual tumors was determined as described in the Materials and Methods. (c) Lungs of individual mice were excised at the end of the experiment and tumor colonies were counted.

Figure 4.

Prolonged survival of tumor-bearing mice by therapy with SN6j and SN6a. 4T1 cells (1.0×10^5 cells) were inoculated into the left mammary gland fat pad on day 0. Mice bearing palpable tumors were divided into 4 groups. Groups of mice were treated with SN6j ($n = 19$), MOPC195v ($n = 19$), SN6a ($n = 18$) or RPC5 ($n = 18$) on day 3, 6, 9 and 16. (a) 10 mice (for each of the MOPC195v and SN6j group) or 9 mice (for each of the RPC5 and SN6a group) from different groups were sacrificed on day 24 and tumor colonies in the lungs from individual mice were counted. (b) Survival of the remaining mice ($n = 9$ for each group) was followed until day 32.

Figure 5.

Effect of SN6j and SN6a on the lung tumor colonies from the intravenously injected 4T1. 4T1 (2.0×10^4 cells/mouse) was inoculated into mice via the tail vein on day 0. Mice were divided into 3 groups ($n = 10$ for each group) and treated with SN6j, SN6a or control IgG (RPC5) on day 3, 6 and 9 after tumor inoculation. Mice were sacrificed on day 16 and tumor colonies in the lungs from individual mice were counted (a). In addition, weight of individual lungs was measured (b).

Figure 6.

Suppression of hepatic metastasis of colon26 colon carcinoma by i.v. administration of anti-ENG mAb or immunotoxin. Colon26 (2×10^4 cells/mouse) was inoculated under capsule of the spleen on day 0, and on day 2 the mice were treated by i.v. administration of a naked anti-ENG mAb, control IgG, immunotoxin (deglycosylated ricin A-chain conjugate), control conjugate (MOPC-dgRA) or control (PBS). Two types of colon26 were used in the experiments. One is the parental clone L0 and the other is a highly metastatic subclone L5 (see Materials and Methods for the details) (a) L0 was used in the experiment. (b) An example of the livers for the SN6k-dgRA-treated group and the control group in (a) is shown. Metastatic colonies are indicated by arrows. (c), (d), L5 was used in these experiments.