Effective anti-angiogenic therapy of established tumors in mice by naked anti-human endoglin (CD105) antibody: Differences in growth rate and therapeutic response between tumors growing at different sites

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Abstract. We investigated anti-angiogenic/vascular targeting therapy of established tumors in immunocompetent mice using an anti-human endoglin (EDG; CD105) monoclonal antibody (mAb) SN6j. SN6j weakly cross-reacted with murine endothelial cells but reacted neither with colon-26 murine colon carcinoma cells nor with 4T1 murine mammary carcinoma cells. Systemic administration of naked (unconjugated) SN6j showed significant growth suppression of established tumors of colon-26 and 4T1 cells in immunocompetent BALB/c mice ($P<0.05$). Moreover, the overall survival rate of SN6j-treated mice was significantly higher than that of control IgG-treated mice ($P<0.01$). During these studies, we found that two different types of tumor formed in BALB/c and immunodeficient SCID mice when three different types of tumor cells (colon-26, 4T1 and MCF-7 human breast cancer cells) were inoculated subcutaneously. One type of tumor grew in the skin-side tissue (i.e., epidermis, corium, or subcutis), and mainly invaded into the corium and epidermis. The other type grew in the muscle-side tissue (i.e., fascia, muscle, or peritoneum/pleura). We termed the former SS tumors and the latter MS tumors. MS tumors grew faster than SS tumors. This differential growth of MS and SS tumors was observed in three different animal models, i.e., colon-26 tumors and 4T1 tumors in BALB/c mice, and MCF-7 tumors in SCID mice. In the therapeutic study of colon-26 and 4T1 tumors with SN6j, MS tumors were less responsive to therapy than SS tumors although SN6j showed significant antitumor efficacy against both tumors ($P<0.05$). The results show that antitumor therapy can yield different therapeutic outcomes depending on the tumor growth sites even for the same tumor. A differential survival between mice with the two types of tumor was also observed when mice were untreated ($P<0.01$).

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Introduction

Anti-angiogenesis of tumors prevents formation of new blood vessels in tumors (1), whereas vascular targeting therapy of tumors attacks the existing neovasculature of tumors to cause tumor cell death from ischemia and extensive hemorrhagic necrosis (reviewed in refs. 2-6). These therapies may have advantages over conventional tumor cell-targeted therapies including chemotherapy and immunotherapy. For instance, tumor vessel-targeted therapies may be able to overcome a major problem associated with other anticancer therapies, i.e., the problems of drug resistance (7,8). In addition, combination of tumor vessel-targeted therapy with other therapies such as chemotherapy and radiotherapy may enhance therapeutic efficacy compared with either therapy alone (reviewed in refs. 9-11). Indeed, the combined use of bevacizumab [Avastin; a humanized anti-vascular endothelial growth factor monoclonal antibody (mAb)] with chemotherapeutic drugs showed statistically significant and clinically meaningful improvement in survival among patients with metastatic colorectal cancer (12).

Endoglin (EDG, CD105), a homodimeric glycoprotein, was initially identified as a human leukemia-associated cell membrane antigen (13,14). Although EDG is not a tumor-specific marker, its expression is relatively restricted (13,15). For instance, anti-EDG mAb SN6 did not show significant reaction with various normal human peripheral blood cells including B cells, T cells, granulocytes, monocytes and erythrocytes (13). Later EDG was detected on activated monocytes/macrophages and activated $CD4^+$ T cells by others (16,17). EDG is a proliferation-associated cell membrane antigen of endothelial (18-21) and leukemia cells (22), and is strongly expressed on the tumor-associated angiogenic vascular endothelium (19-21,23). In addition, EDG is essential for angiogenesis (24,25) and a component of the transforming growth factor (TGF)-ß receptor complex (26). Recently, the EDG gene was reported to be one of the genes that showed higher expression in circulating endothelial cells from cancer patients than those from healthy donors (27). Some of the anti-EDG mAbs generated in our laboratory weakly cross-reacted

with mouse endothelial cells and certain anti-EDG immunoconjugates (immunotoxins and radioimmunoconjugates) showed both anti-angiogenic activity (20,28) and vascular targeting activity (29) in animal studies. However, these immunoconjugates also showed undesirable side effects in mice when the dose was not chosen carefully. For instance, LD_{50} value of SN6f immunotoxin and SN6j immunotoxin in BALB/c mice was 14.4 and 16.6 μg/g body weight, respectively, by i.v. injections (20,29). Therefore, the therapeutic windows of these immunotoxins were relatively narrow. In contrast to immunotoxicins, naked (unconjugated) anti-EDG mAbs showed no significant toxicity in a dose-escalation study in mice.

In the present study, we examined the antitumor activity of SN6j, which shows weak cross-reactivity with mouse endothelial cells (29), against the tumor growth in immunocompetent mice. The naked mAb showed statistically significant antitumor efficacy against established tumors. During the present study, we discovered that two different forms of tumor appear when tumor cells are inoculated s.c. into mice; one type grows in the skin-side tissue (i.e., epidermis, corium, or subcutis; termed SS tumors), and the other type grows in the muscle-side tissue (i.e., fascia, muscle, or peritoneum/pleura; MS tumors). Formation of the two types of tumor was observed when three different tumor cells (colon-26 murine colon adenocarcinoma cells, 4T1 murine mammary carcinoma cells and MCF-7 human breast cancer cells) were inoculated s.c. into immunocompetent BALB/c mice or immunodeficient SCID mice. SS tumors and MS tumors showed different growth patterns and different therapeutic responses. These differences may skew test results of anticancer agents against s.c. tumors. These findings are important because the s.c. tumor models are the most commonly used tumor models in the *in vivo* evaluation of anticancer agents. The anti-EDG mAb showed antitumor efficacy against both SS and MS tumors although it was to a higher degree against SS tumors.

Materials and methods

Cells and animals. The colon-26 murine colon adenocarcinoma cell line was kindly provided by Dr Tateshi Kataoka (Cancer Chemotherapy Center, Tokyo, Japan) and cultured in a monolayer in RPMI-1640 media containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. The 4T1 murine mammary carcinoma cell line was obtained from American Type Culture Collection (Rockville, MD) and cultured in a monolayer in RPMI-1640 media supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. The MCF-7 human breast cancer cell line was obtained and cultured as described previously (20). The KM-3 and MOLT-4 human leukemia cell lines were cultured as described previously (13). All cells were cultured in a humidified atmosphere with 5% CO_2 at 37˚C.

Five- to six-week-old BALB/c and SCID mice were obtained from National Cancer Institute (Bethesda, MD) and Roswell Park Cancer Institute (RPCI). They were used for experiments after being acclimated for at least a week. They were supplied autoclaved food and water *ad libitum*. All handling of the mice was performed in a laminar flow hood.

Monoclonal antibodies and control IgG. An anti-EDG mAb SN6j that weakly cross-reacts with mouse endothelial cells was generated in our laboratory (29). An isotype-matched murine control IgG MOPC 195 variant (IgG1-κ) was also generated in our laboratory (13). SN6j and MOPC 195v were individually sterilized by filtering through a Millex-GV filter (0.22 μm; Millipore, Billerica, MA) in a laminar flow hood. The sterilized solutions were diluted with sterilized PBS containing mouse serum albumin (final concentration, 0.05%).

Cellular radioimmunoassay (RIA). Cellular RIA was used to determine the reactivity of SN6j with colon-26 and 4T1 cells. Details of the assay were described previously (30).

Histological staining. Tissue specimens including tumors were obtained from mice with established tumors. The stained tissues included epidermis, corium, subcutaneous tissue, muscle layer, and peritoneum or pleural, and were fixed with 10% neutral-buffered formalin immediately after surgical removal. The fixed tissues were processed according to standard procedures, and then embedded in paraffin. For hematoxylin-eosin staining, 5-μm paraffin sections were placed on glass slides and dried in a 60˚C oven for 1 h. After the slides were cooled to room temperature, they were deparaffinized with xylene, and rehydrated with graded ethanols, and distilled water. The sections were then stained with Harris hematoxylin (Poly Scientific, Bay Shore, NY) and alcoholic eosin Y (Fisher Scientific Company, Fair Lawn, NJ) solutions, dehydrated with ethanols, cleared with Histo-clear II (National Diagnostics, Atlanta, GA), and coverslipped with an acrylic mounting medium. Normal tissues obtained from tumor-free normal mice were also stained in the same manner.

Transplantation of tumor cells into BALB/c mice or SCID mice. Cultured colon-26 and 4T1 cells were harvested using Hanks solution containing 3 mM EDTA and 25 mM HEPES, washed twice and then suspended in PBS, pH 7.2. A portion (0.1 ml) of the cell suspensions containing the desired number of cells was inoculated s.c. into the left flanks of BALB/c mice using a 30G1/2 needle (BD 30G1/2 PrecisionGlide Needle; Becton Dickinson, Franklin Lakes, NJ) to establish s.c. tumors. Transplantability of colon-26 and 4T1 cells in BALB/c mice was investigated by performing dose-escalation titration experiments using 0.625x, 1.25x, 2.5x, and $5x10^5$ colon-26 cells (n=7 in each group) and 0.2x, 1x, and $5x10^5$ 4T1 cells (n=6) in 0.1 ml PBS. MCF-7 human breast cancer cells were inoculated s.c. into SCID mice as described previously (20).

Determination of the growth-site of tumors. We found that two different types of tumor appeared when tumor cells were injected to make s.c. tumors in mice; one was growing in the skin-side tissue (i.e., epidermis, corium, or subcutis), and the other was growing in the muscle-side tissue (i.e., fascia, muscle, or peritoneum/pleura). The former and latter types of tumor were termed the SS type and the MS type, respectively. To determine where a tumor was growing, the skin just above the tumor was slid after appearance of a distinct tumor. If the tumor was fixed in the skin and slid together with the skin, the tumor was identified as the SS type. If the tumor was

fixed in the deeper site and didn't move with the skin, the tumor was identified as the MS type. The growth rate of tumors was substantially different between the SS type and the MS type tumors. Therefore, at the onset of experiments, we not only distributed mice with tumors of a similar size evenly into different groups but also distributed mice with SS type and MS type tumors evenly into different groups. The tumors in a small number of mice did not belong to either the SS type or MS type, i.e., they were fixed in neither skin nor a deeper site. These tumors were termed intermediate type, the IN type.

Therapy of mice bearing colon-26 and 4T1 tumors. For the colon-26 tumor model, BALB/c mice were inoculated s.c. with $1.25x10^5$ colon-26 cells as determined in the earlier titration experiment. Five days after tumor inoculation, mice with distinct tumors were selected and divided into groups according to tumor volume, tumor growth-site, and body weight. Groups of mice bearing established tumors were treated by i.v. administration of 0.6 μg/g body weight of SN6j in 0.2 ml PBS containing 0.05% mouse serum albumin via the tail vein. The mAb dose was chosen based on the earlier dose-dependent titration experiment. As a control, the mice were treated by i.v. administration of 0.6 μg/g body weight of an isotype-matched control IgG (MOPC 195v; IgG1-κ) in 0.2 ml PBS containing 0.05% mouse serum albumin via the tail vein. The treatment was repeated every 3 days. For the 4T1 tumor model, 4T1 cells ($1.0x10^5$ cells in 0.1 ml PBS/mouse) were inoculated s.c. Mice with distinct tumors were divided into groups as described above. Groups of mice bearing established tumors were treated by i.v. administration of 1.8 μg/g body weight of SN6j or control IgG. The treatment was performed at 3-day intervals for the first three injections and at a 7-day interval for the fourth injection.

Follow-up of treatment efficacy. During the treatment, mice were monitored daily for morbidity. Mice were weighed every other day using an electronic balance (Scout Pro 202, Ohaus Corp., NJ). Tumor size was measured every other day using an electronic digital caliper (Ultra-Cal IV, Ted Pella, Inc., CA) that was connected to a computer using GageWedge software (Fred V. Fowler Company, Inc., Newton, MA). The measured tumor diameters were converted to tumor volume using Excel 2003 and the following formula: V= length x width x height x π/6.

MCF-7 tumors in SCID mice. To determine the relationship between growth site and growth rate of tumors in an additional s.c. tumor model set, we used the MCF-7 human breast tumor cell line in SCID mice. MCF-7 cells ($8x10^6$ cells in 0.1 ml PBS/mouse) were injected s.c. into the left flanks of SCID mice using a 25G5/8 needle (BD 25G5/8 PrecisionGlide Needle; Becton Dickinson) to generate s.c. tumors. The mice were monitored and tumor was measured twice a week for 70 days after tumor inoculation.

Statistical analysis. 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 log-rank test (StatView software, version 5).

Results

Lack of reactivity of SN6j with colon-26 and 4T1 cells. Potential reactivity of SN6j with colon-26 and 4T1 cells was tested by a cellular RIA (30). In the assay, EDG-positive KM-3 human B-lineage leukemia cells and EDG-negative MOLT-4 human T leukemia cells (13,20) were included as a positive and a negative control, respectively. In addition, an isotype-matched control IgG was included as another negative control. The binding value of SN6j to colon-26 (140±24 cpm) was similar to that of SN6j to MOLT-4 (161±50 cpm) while the value was much lower than that of SN6j to KM-3 (1,464±151 cpm). The binding values of the control IgG to colon-26, KM-3 and MOLT-4 were similar, i.e., 185±40, 177±45 and 139±22 cpm, respectively. The results indicate that the observed binding value (140±24 cpm) of SN6j to colon-26 represents a background value of the assay and SN6j does not react with colon-26. Similarly, results of cellular RIA showed that SN6j does not react with 4T1. Previously we showed that SN6j weakly cross-reacts with murine endothelial cells (29).

Dose titration of colon-26 and 4T1 cells in BALB/c mice. Two-fold serial increments (i.e., 0.625x 1.25x, 2.5x, and $5x10^5$ in 0.1 ml PBS) of colon-26 cells were injected into the left flanks of BALB/c mice to establish s.c. tumors (n=7 in each group). We followed the tumor size for 20 days after inoculation, and distinct tumors appeared in all of the mice except one which was inoculated with the lowest dose, i.e., $0.625x10^5$ cells (data not shown). Therefore, the dose of $1.25x10^5$ cells in 0.1 ml PBS was used in the following colon-26 studies. In the case of 4T1, 5-fold serial increments (i.e., 0.2x, 1x, and $5x10^5$ cells in 0.1 ml PBS) of 4T1 cells were injected s.c. into groups of mice (n=6) as described above. Distinct tumors appeared in all of the group 2 and group 3 mice, but tumors were not detected in any of the group 1 mice. Therefore, the dose of $1x10^5$ cells was used in the subsequent 4T1 studies.

Two different forms of tumors. We found that two different types of tumor appeared when tumor cells (i.e., colon-26, 4T1 or MCF-7 cells) were inoculated into mice to generate s.c. tumors. One type of tumor was fixed in the skin and slid together with the skin, and the other type of tumor was fixed in the deeper site and didn't move with the skin. The former type of tumor usually makes an ulcer in the skin, whereas the latter rarely does. Interestingly, the growth rate of the tumors of the latter type was generally faster than that of the former type (Fig. 1A and B). We examined the histological differences between the two types of tumor in mice, and the results are shown in Fig. 1C and D. The former type of tumor grew and invaded the skin-side tissues (SS tissue; i.e., epidermis, corium, or subcutis) while it did not invade the muscle-side tissues (MS tissue; i.e., fascia, muscle, peritoneum, or pleura) and MS tissues were intact. On the other hand, the latter type grew and invaded the MS tissues and the SS tissues of this type were rarely invaded by tumors. Therefore, we named the former type SS type tumor (SS tumor) and the latter type MS type tumor (MS tumor). A small number of tumors were fixed in neither skin nor a deeper site. This type of tumor was termed intermediate type (IN type). Because the number of

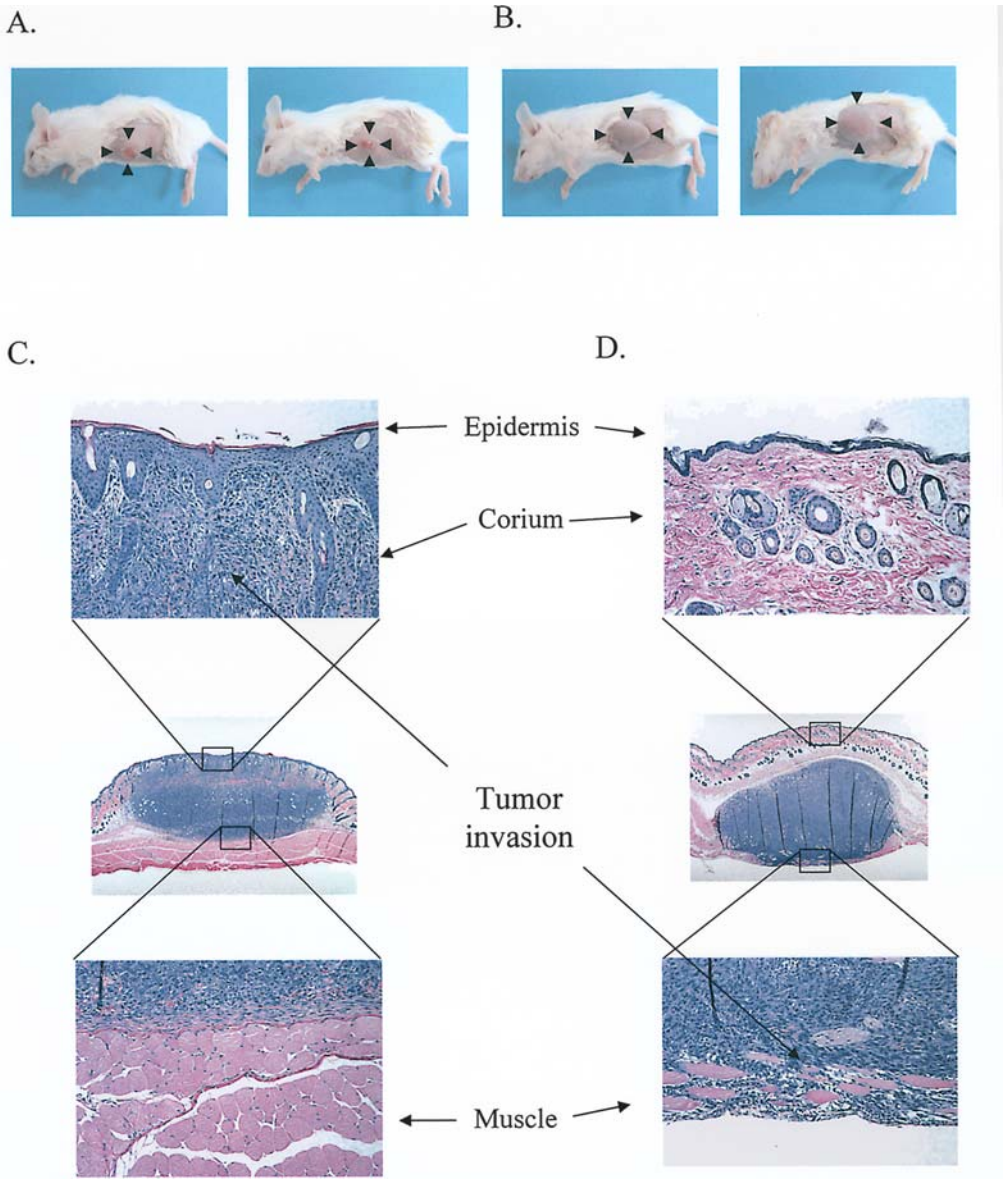

Figure 1. Formation of two different types of tumor by s.c. inoculation of colon-26 cells into BALB/c mice. One type of tumor mainly grew in the skin-side tissue (SS tissue), and the other type mainly grew in the muscle-side tissue (MS tissue). A, examples of the SS type tumors (SS tumors) of colon-26 20 days after tumor cell inoculation ($1.25x10^5$ cells/mouse). B, examples of the MS type tumors (MS tumors) of colon-26 20 days after tumor cell inoculation ($1.25x10^5$ cells/mouse). MS tumors grew more rapidly than SS tumors as seen in A and B (see also Figs. 2C, 3B and 4). SS tumors usually made ulcers in the skin, whereas MS tumors rarely made ulcers. C, hematoxylin and eosin (H&E) staining of an SS tumor 7 days after tumor cell inoculation ($1.25x10^5$ cells/mouse). Invasion of the tumor is observed in subcutaneous tissue, corium, and epidermis, and the tumor is less invasive to the muscle side. D, H&E staining of an MS tumor 7 days after tumor cell inoculation ($1.25x10^5$ cells/mouse). The corium and epidermis are intact compared with the muscle side tissue which is invaded by the tumor. Original magnification, x4 (the middle panels of C and D) or x200 (the top and bottom panels of C and D).

mice bearing the IN type tumors (IN tumors) was quite small in the colon-26 tumor model (e.g., 4.0% of the colon-26-inoculated mice), the mice with the IN tumors were not used in the present EDG-targeted therapy. The test results of 4T1 were very similar to those of colon-26 (data not shown).

Antitumor efficacy of SN6j against colon-26 tumor and 4T1 tumor. Colon-26 cells ($1.25x10^5$ cells/mouse) were injected s.c., and the mice were left untreated until distinct palpable tumors appeared. Mice with distinct tumors were selected and divided into two groups (n=18 each) and were treated by i.v. administration of SN6j or an isotype-matched control IgG. The dose of 0.6 μg SN6j/g body weight was chosen based on the titration experiment (see above). SN6j showed significant antitumor efficacy compared with the isotype-matched control IgG as evaluated by measuring the tumor size ($P<0.05$) (Fig. 2A). Furthermore, four of the eighteen mice (22.2%) in the SN6j-treated group and twelve of the eighteen mice (66.7%) in the control group died or were moribund on or before day 25 after tumor inoculation (Fig. 2B). Analysis of the results showed that the overall survival rate of the SN6j-treated group was significantly higher than that of the control group ($P<0.01$) (Fig. 2B). The results demonstrate that SN6j is effective for tumor suppression based on the criteria of either tumor size or survival. Of the above eighteen tumor-bearing mice in the SN6j-treated group and the control group, 12 mice bore SS tumors while the remaining 6 mice bore MS tumors. In the SN6j-treated group, the size of MS

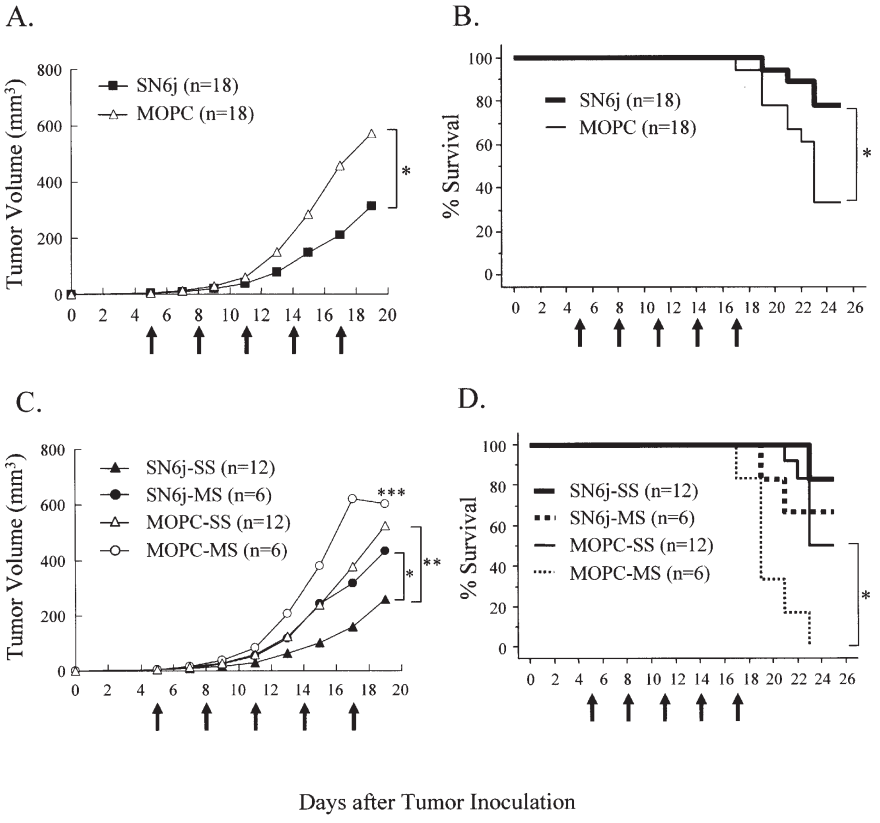

Figure 2. Antitumor efficacy of a naked anti-EDG mAb SN6j against colon-26 tumors. Colon-26 cells ($1.25x10^5$ cells/mouse) were injected s.c. into BALB/c mice. Mice bearing distinct palpable tumors were divided into 2 groups (n=18 in each group), and treated by i.v. administration of 0.6 μg/g body weight) of SN6j or an isotype-matched control IgG (MOPC 195 variant, IgG1-κ). Each group consisted of 12 mice with SS tumors and 6 mice with MS tumors. A, average size of the tumors of mice during treatment with SN6j or control IgG (n=18 in each group). There is a significant difference in tumor size between the two groups (P<0.05). B, difference in survival between SN6j-treated group and control IgG-treated group. The SN6j-treated mice showed significantly better overall survival than the control IgG-treated group (P<0.01). C, comparison in tumor size between mice bearing SS tumors and MS tumors. The average size of MS tumors was larger than that of SS tumors in both SN6j-treated group and control group. The difference was statistically significant (P<0.05) for the SN6j-treated group. In addition, the SN6j-treated group showed significantly smaller tumor size than the control IgG-treated group (**P<0.05). The average size of MS tumors in the control IgG-treated group stopped increasing after day 16 because MS tumors in two moribund mice in this group became shrunk after day 16 (***). D, difference in survival between mice bearing SS and MS tumors. Mice bearing SS tumors showed better overall survival than those with MS tumors (P<0.001 for the control IgG-treated group). Arrows in the figure indicate i.v. injections of SN6j or the isotype-matched control IgG.

tumors was significantly larger than that of SS tumors (P<0.05) (Fig. 2C). Furthermore, two of the twelve mice (16.7%) with SS tumors and two of the six mice (33.3%) with MS tumors died or were moribund on or before day 25 in the SN6j-treated group (Fig. 2D). Thus, both results of the tumor growth and mouse survival indicate that SS tumors are more responsive than MS tumors to therapy with SN6j.

In the case of the 4T1 tumor model, $1x10^5$ 4T1 cells were injected s.c. into individual BALB/c mice. Mice bearing distinct palpable tumors were divided into 2 groups (n=22 in each group), and treated by i.v. administration of 1.8 μg/g body weight of SN6j or an isotype-matched control IgG. Each group consisted of 12 mice with SS tumors and 10 mice with MS tumors. SN6j showed significant antitumor efficacy compared with the isotype-matched control IgG as evaluated by measuring the tumor size (P<0.01) (Fig. 3A). Of the SN6j-treated mice, the size of MS tumors was significantly larger than that of SS tumors (P<0.05) (Fig. 3B).

These data demonstrate that systemic (i.v.) administration of naked mAb SN6j is effective for suppression of both SS and MS tumors although SN6j is more effective against SS tumors than MS tumors.

Differences in tumor growth between SS and MS tumors in three animal models. To understand the factors involved in the different therapeutic response between SS and MS tumors, we analyzed tumor growth in untreated mice. In the control IgG (MOPC 195 variant)-treated group, the average size of MS tumors was larger than that of SS tumors for both colon-26 and 4T1 tumors (Fig. 2C). Moreover, in the control IgG-treated group of mice bearing colon-26 tumors, six of the twelve mice (50%) bearing SS tumors and six of the six mice (100%) bearing MS tumors died or became moribund on or before day 25 (Fig. 2D). The results demonstrate that the overall survival of the mice with SS tumors was significantly higher than that of the mice with MS tumors (P<0.001; Fig. 2D).

The above observation of BALB/c mice bearing colon-26 and 4T1 murine tumors was extended to studies of SCID mice bearing MCF-7 human breast tumors (Fig. 4). The average size of MS tumors was significantly larger than that of SS tumors (P<0.05; Fig. 4). Generation of the s.c. tumors was independently performed by two physician scientists (Masanori Tsujie and Shima Uneda) who have had extensive experience with s.c. injections of substances into patients and mice, and they obtained consistent results for MS and SS

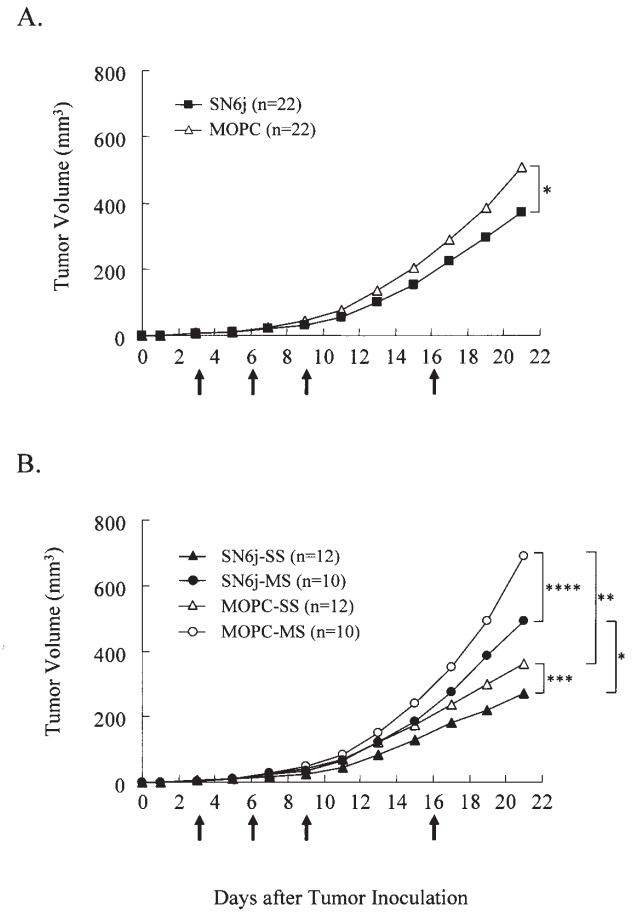

Figure 3. Antitumor efficacy of SN6j against 4T1 tumors. 4T1 cells ($1x10^5$ cells/mouse) were injected s.c. into BALB/c mice. Mice bearing distinct palpable tumors were divided into 2 groups (n=22 in each group), and treated with SN6j (1.8 μg/g BW) or an isotype-matched control IgG (MOPC 195 variant, 1.8 μg/g BW). Each group consisted of 12 mice with SS tumors and 10 mice with MS tumors. A, average size of the tumors during treatment with SN6j or control IgG (n=22 in each group). There is a significant difference in tumor size between the two groups ($P<0.01$). B, comparison in tumor size between mice bearing SS tumors and MS tumors. The average size of MS tumors was significantly larger than that of SS tumors in both the SN6j-treated group and control group ($^{*,**}P<0.05$). In addition, the SN6j-treated group showed significantly smaller tumor size than the control IgG-treated group ($^{***,****}P<0.05$).

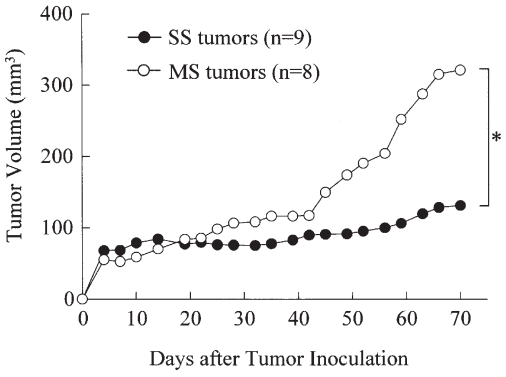

Figure 4. Generation of two types of tumor by s.c. inoculation of MCF-7 into SCID mice. MCF-7 human breast cancer cells were injected into SCID mice to generate s.c. tumors. The SS and MS tumors of MCF-7 appeared in the SCID mice as in the cases of colon-26 and 4T1 in BALB/c mice. Again the average size of MS tumors is significantly larger than that of SS tumors in the SCID mice ($P<0.05$).

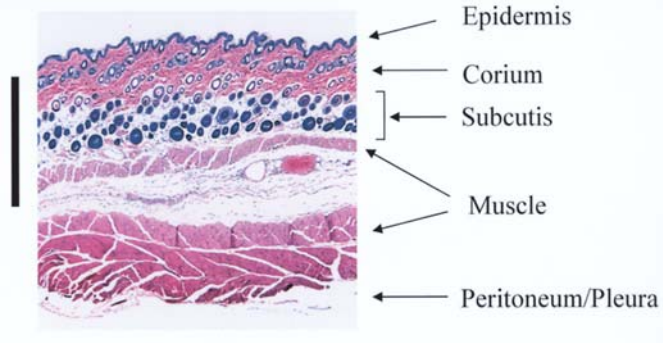

Figure 5. A vertical section of the left flank of a normal BALB/c mouse. Thickness of subcutis of the subcutaneous tissue is approximately ≤500 μm. Scale bar, 1 mm.

tumors. In addition, consistent results from three animal models demonstrate that the growth rate of MS tumors is faster than that of SS tumors in mice and suggest that this difference in the tumor growth may be a major factor for the different therapeutic response between MS and SS tumors.

Discussion

Most of the current drugs used in systemic therapy are cytotoxic chemotherapeutics that are designed to inhibit or kill rapidly dividing cells directly. Therefore, these drugs also damage the normal dividing cells of rapidly regenerating tissues such as bone marrow, and often cause toxic side effects, i.e. myelosuppression. Moreover, many cancer cells intrinsically have diverse genetic defects and instability that may lead to acquired drug resistance. These and other problems associated with tumor-cell targeted therapy may be circumvented by anti-angiogenic and vascular targeting therapy of cancer (reviewed in refs. 5,9). In addition, a single agent developed for anti-angiogenic and/or vascular targeting therapy could be applied to most types of solid tumors.

One approach to the anti-angiogenic and vascular targeting therapy is antibody-based targeting of tumor vasculature. In order to effectively apply anti-angiogenic/vascular targeting therapy to cancer, it is imperative to develop appropriate reagents that selectively destroy tumor-associated vasculature without severely damaging the vasculature and other vital components of normal tissues. Endothelium in normal adults is quiescent and the turnover of these cells is very low (i.e. thousands of days) (31). However, the same endothelial cells can undergo rapid proliferation during spurts of angiogenesis. EDG is a cell membrane antigen, the expression of which is relatively restricted (13) and is stronger on tumor endothelium than on normal endothelium (19-21,23). Our anti-EDG mAb showed minimal reactivity with normal human bone marrow cells (13).

In the animal model studies, we selected a few anti-human EDG mAbs that show weak but measurable cross-reactivity with mouse endothelial cells (20,28,29,32). We have been able to effectively target EDG on tumor vasculature in SCID mice by using immunoconjugates (immunotoxins and radioimmunoconjugates) of these anti-EDG mAbs (20,28,29).

These immunoconjugates showed anti-angiogenic activity by preventing formation of new blood vessels and inhibiting formation of new tumors (20,28). They also showed vascular targeting activity by suppressing the growth of established tumors (29). However, anti-EDG immunotoxins showed significant undesirable side effects in mice. In contrast, no toxicity/side effects were detected for naked anti-EDG mAb SN6j in a dose escalation study. To overcome weak cross-reactivity with mouse endothelial cells of these anti-EDG mAbs in mouse models, we used human skin/SCID mouse chimeras in which tumor growth was supported by a mixture of human and mouse blood vessels (32). In this model, naked anti-EDG mAbs were able to show significant vascular targeting activity against established human tumors in the chimeras (32). Nevertheless, it is very time-consuming and laborious to generate and utilize the chimeras for therapeutic studies. In the present study, therefore, we examined the effect of one of the naked anti-EDG mAbs against the tumor growth in immunocompetent mice.

The present results show that systemic (i.v.) administration of a naked anti-EDG mAb SN6j not only suppresses the growth of established tumors but also improves the survival of immunocompetent mice bearing established tumors. SN6j should exert stronger antitumor efficacy in patients than in mice because SN6j reacts much more strongly with proliferating human endothelial cells than with the mouse counterpart (29). Indeed, our previous test results using human skin/SCID mouse chimeras showed that SN6j therapy resulted in a stronger suppression of angiogenic human vessels in the tumors compared with murine vessels in the same tumors (32). To facilitate clinical application of the anti-EDG mAbs, we generated a recombinant human/mouse chimeric mAb termed c-SN6j from SN6j. A dose escalation study of c-SN6j was performed in non-human primates to evaluate the pharmacokinetic parameters, immunogenicity and potential toxicity of c-SN6j (33). The results are very encouraging.

In this study, we found that two types of tumor are generated by s.c. inoculation of colon-26 murine colon adenocarcinoma cells into BALB/c mice; one type grows mainly in the skin-side tissue (SS type), and the other type grows mainly in the muscle-side tissue (MS type). The SS tumors usually make ulcers in the skin, whereas the MS tumors rarely do. This observation was confirmed by s.c. inoculation of 4T1 murine mammary carcinoma cells into BALB/c mice and MCF-7 human breast cancer cells into SCID mice. Furthermore, the observation was independently made by two physician-scientists (Masanori Tsujie and Shima Uneda) who have had extensive experience with s.c. injections of substances into patients and mice. In the conventional definition, s.c. tumors should refer to SS tumors.

To understand the reasons for the generation of the two types of tumor by s.c. inoculation of tumor cells into mice, we examined the thickness of subcutaneous tissue and the needles used for the inoculation of tumor cells. The thickness of subcutis in the left flank of a normal BALB/c mouse is approximately ≤500 μm (Fig. 5). The thickness of 30G1/2 and 25G5/8 needles is approximately 300 and 500 μm, respectively. These results indicate that the subcutis region is too narrow to precisely inject a cell suspension into subcutis by using these needles. The cells which are injected into a deeper site and/or cell leak from subcutis into a deeper site will form MS tumors. The present findings are important because s.c. tumor models of mice are the most widely used animal models for evaluation of antitumor agents *in vivo*.

Test results of antitumor agents in s.c. tumor models will be complicated by the formation of SS and MS tumors in different ratios under different circumstances. The ratio will be influenced by several factors including needle size, injection techniques and mouse strains. Therefore, careful examination of the tumors including ratio of SS tumors to MS tumors before analysis of the data will be important. MS tumors grow faster than SS tumors, and this difference was consistently observed in three different animal models, i.e., colon-26 murine colon tumors in BALB/c mice, 4T1 murine mammary tumors in BALB/c mice and MCF-7 human breast tumors in SCID mice. Thus, the generation of different types of tumor in an s.c. inoculated tumor is probably a common incidence in s.c. tumor models in mice.

The antitumor efficacy of EDG-targeted therapy was more pronounced against SS tumors than against MS tumors. The present results suggest that a major factor for this difference is a difference in the tumor growth rate, i.e., the faster growth of MS tumors compared with SS tumors. An additional factor for the difference in the therapeutic response will be different microenvironments of MS and SS tumors. Over the past decade, it has become clear that the growth of tumors is influenced by tumor microenvironments (reviewed in refs. 34,35). Furthermore, endothelial cells from different organs were found to possess distinct growth factor profiles (36). Microenvironments of MS tumors and SS tumors are expected to differ. Different characteristics of endothelial cells and/or different expression levels of angiogenic factors will influence the outcome of anti-angiogenic/vascular targeting therapy of tumors.

Our results collectively indicate that we need to take into consideration the likelihood that s.c. inoculated tumors will consist of a mixture of different forms of tumor, i.e., SS, MS and intermediate type tumors, when we use s.c. tumor models to evaluate the efficacy of antitumor agents. Therefore, we may need to divide the tumor-bearing mice into groups of mice bearing SS or MS tumors before we initiate therapy of s.c. tumors using an antitumor agent(s). Otherwise, we may be unable to correctly interpret the test results.

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