

Combined Anti-Fetal Liver Kinase 1 Monoclonal Antibody and Continuous Low-Dose Doxorubicin Inhibits Angiogenesis and Growth of Human Soft Tissue Sarcoma Xenografts by Induction of Endothelial Cell Apoptosis¹

Lianglin Zhang, Dihua Yu,² Daniel J. Hicklin, Jonathan A. F. Hannay, Lee M. Ellis, and Raphael E. Pollock²

Department of Surgical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 [L. Z., D. Y., J. A. F. H., L. M. E., R. E. P.], and Department of Immunology, ImClone Systems Inc., New York, New York 10014 [D. J. H.]

ABSTRACT

Vascular endothelial growth factor (VEGF) and VEGF receptor 2 [fetal liver kinase 1 (Flk-1)/kinase insert domain-containing receptor] have been shown to play a major role in tumor angiogenesis. In this study, we investigated whether anti-Flk-1 monoclonal antibody DC101 could therapeutically inhibit growth and angiogenesis of human soft tissue sarcoma, and we explored its capacity to enhance the tumoricidal effects of doxorubicin. Treatment of well-established leiomyosarcoma SKLMS-1 and rhabdomyosarcoma RD xenografts in severe combined immunodeficient mice with DC101 resulted in significant antitumor activity. In a parallel study, we compared tumor inhibition with continuous low-dose “antiangiogenic” schedule versus once-every-2-weeks high-dose standard schedule of doxorubicin. We found that continuous low-dose treatment inhibited the tumor growth of RD xenografts about 46.5% of that with standard-schedule treatment, but that continuous low-dose treatment did not inhibit the tumor growth of SKLMS-1 xenografts. Notably, combined DC101 and continuous low-dose doxorubicin resulted in more effective growth inhibition of SKLMS-1 and RD xenografts than has been observed with any agent alone in a long-term s.c. tumor xenograft model. The combination therapy was associated with no additional toxicity to the host animal compared with low-dose doxorubicin alone. Histological examination of xenografts showed significantly reduced microvessel counts in the tumors given combined therapy compared with the tumors given either agent alone. These results are consistent with an enhanced inhibition of angiogenesis *in vivo* by combined DC101 and doxorubicin using Matrigel plug assay. Additionally, DC101 plus doxorubicin directly exerted enhanced inhibitory effects on endothelial cell migration, proliferation, and tube-like formation *in vitro*. Furthermore, the combination induced an enhanced apoptosis of endothelial cells that was associated with an increase of caspase-3 activity. Thus, the inhibition of angiogenesis and induction of endothelial cell apoptosis are likely important mechanisms for the antitumor activity of combined DC101 and doxorubicin. Collectively, our data suggested that anti-VEGF receptor 2 in combination with continuous low-dose doxorubicin may provide a new therapeutic approach for human soft tissue sarcoma in the clinic.

INTRODUCTION

Angiogenesis is defined as the growth of new blood vessels from preexisting ones. This process plays a key role in numerous human diseases, such as malignant tumor progression (1, 2). Among the most substantial mediators of angiogenesis are the VEGFs³ and their receptors (3). VEGF, formerly named vascular permeability factor

(VPF) or vasculotropin (VAS) is an endothelial cell-specific mitogen, which is secreted as a M_r 45,000 protein consisting of two subunits that do not induce cell proliferation in other cell types (4). The physiological importance of VEGF and VEGFRs in blood vessel formation has been clearly demonstrated in gene knockout experiments, which demonstrated that targeted deletion of the *VEGF* gene (5), *Flk-1* gene (6), and *Flt-1* gene (7) in mice resulted in embryonic lethal phenotypes. VEGF is abundantly expressed in a variety of human tumors and appears to be a crucial mediator of tumor angiogenesis (8–10). VEGF may be a marker of tumor endothelium (11). VEGF expression is strongly up-regulated by hypoxia and oncogenes that are tightly associated with rapidly growing tumors (12, 13). VEGFRs have been shown to be involved in the regulation of tumor angiogenesis in various tumors (14). The important role of VEGF/VEGFRs in tumor angiogenesis was directly demonstrated in studies in which anti-VEGF neutralizing antibody significantly reduced tumor angiogenesis and growth, and expression of a dominant-negative Flk-1 receptor led to marked inhibition of glioma xenografts in nude mice (15).

Antiangiogenesis therapy is aimed at cutting the blood supply of a tumor, an effect that may actually inhibit or destroy the tumor itself. One of the antiangiogenesis therapeutic strategies is to suppress the activity of the major angiogenic regulators, such as VEGF/VEGFR, which is based on their important role in tumor angiogenesis (16). Antiangiogenesis therapies that target VEGF/VEGFR activity, such as antisense against *VEGF* (17), anti-VEGF mAb (18), soluble VEGFR (19), a Flk-1/kinase insert domain-containing receptor kinase inhibitor (20), and anti-VEGFR mAb (21) have in fact inhibited tumor growth. Another antiangiogenic approach is antiangiogenic chemotherapy, which emerged recently and is currently in development (22). Chemotherapy drugs designed to kill tumor cells in patients also act on the tumor endothelium to inhibit tumor angiogenesis (23). However, patients receiving chemotherapeutic agents at the maximum tolerated doses often require extended treatment-free periods for recovery. During the recovery period, dividing endothelial cells within the tumor can also recover from chemotherapy toxicity to resume angiogenesis (24). Two recent reports (24, 25) have shown that continuous low-dose administration of chemotherapy agents or antiangiogenic-schedule chemotherapy inhibits growth of drug-resistant tumor xenografts by inhibiting angiogenesis and preventing the vascularization of tumors without overt toxicity. Moreover, these frequently administered chemotherapeutic agents may sensitize the tumor endothelium to other antiangiogenic therapies, such as TNP-470 or anti-VEGFR antibody, and the combined therapy inhibits tumor growth much more effectively than chemotherapy alone. This newly emerged concept has led to extensive interest in reexamining current chemotherapeutic drugs in antiangiogenic-schedule approaches (22, 26).

Human STSs are a heterogeneous group of rare malignant tumors of putative mesenchymal origin (27). Although aggressive multimodality treatments have been used to manage this disease, the 5-year

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² To whom requests for reprints should be addressed, at Department of Surgical Oncology, Box 107 (D. Y.) or Box 444 (R. E. P.), The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 792-3636 (D. Y.) or (713) 792-6928 (R. E. P.); Fax: (713) 794-4830 (D. Y.) or (713) 792-0722 (R. E. P.).

³ The abbreviations used are: VEGF, vascular endothelial growth factor; CM, conditioned medium; DMEM/F-12 medium, 1:1 mixture of Ham's F-12 nutrient mixture and DMEM; MluE, murine lung endothelial (cell); HUVEC, human umbilical vein endothelial cell; flk-1, fetal liver kinase 1; mAb, monoclonal antibody; SCID, severe combined

immunodeficiency; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; VEGFR, VEGF receptor; STS, soft tissue sarcoma.

survival rate has stagnated at approximately 50% for the past several decades (28). Surgery, with or without radiation therapy, is the mainstay of treatment for early-stage disease in most types of STS. Despite surgical excision, however, more than half of the sarcoma patients die of the disease. For advanced or metastatic STSs, chemotherapy is the current treatment of choice, but no new therapies have been introduced since the inception of doxorubicin-based multichemotherapy drug regimens *e.g.*, combination of doxorubicin and ifosfamide. However, these regimens usually result in increasing toxicity, rapid development of resistance, and no significant survival advantage (29). Thus, new therapeutic strategies are urgently needed to change the limited response rates and severe side effects, *e.g.*, bone marrow suppression and cardiac toxicity from conventional chemotherapy. One possibility is suggested by the highly vascularized nature of STS (30). Brisk neovascularization at the sarcoma: normal tissue interface is macroscopically observable and occasionally can even compromise surgical resectability. Large central cores of hypoxic tumor necrosis are seen as these malignancies proliferate and outstrip their blood supply. A recent report (31) and our previous study (32) suggested that angiogenic factors, *e.g.*, VEGF and basic fibroblast growth factor play a very important role in human STS growth and angiogenesis. Therefore, an emerging awareness of tumor angiogenesis has prompted much interest in exploiting this component of the malignant phenotype as a potential therapeutic target.

Our aim here was to study the therapeutic effect of anti-Flk1 mAb DC101 (21) on growth and angiogenesis of human STS and to explore its capacity to enhance the tumoricidal effects of doxorubicin given on an antiangiogenic schedule. We found that DC101, as a single agent, significantly inhibited the growth of human STS xenografts. We also observed an additive inhibition of growth and angiogenesis in human STS xenografts by combined DC101-doxorubicin therapy.

MATERIALS AND METHODS

Animals. Female SCID mice weighing from 18 to 22 g were obtained from Taconic Farm (Germantown, NY). Animals received humane care in accordance with the Animal Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals." The Institutional Animal Care and Use Committee at The University of Texas M. D. Anderson Cancer Center approved the experiments.

Cell Culture. Human SKLMS-1 leiomyosarcoma and RD rhabdomyosarcoma cells from American Type Culture Collection (ATCC, Rockville, MD) were cultured in DME/F-12 medium with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY) in 5% CO₂ and 95% air at 37°C. The MluE and HUVEC cell lines from ATCC were cultured in F12K medium (Life Technologies, Inc., Grand Island, NY) with 10% fetal bovine serum, 100 unit/ml heparin, and 75 µg/ml of endothelial cell growth supplement (ECGS; SIGMA, Saint Louis, MI) in 5% CO₂ and 95% air at 37°C. Cells were passaged by treatment with a solution containing 0.25% trypsin and 1 mM EDTA (Life Technologies, Inc.) when cells reached 80% confluence.

Preparation of CM. Sarcoma cell CM was prepared as described previously (33). Briefly, cultures of the various sarcoma cell lines were rinsed twice with serum-free DMEM/F12 and cultured in 10 ml of serum-free DMEM/F12. One day later, the culture medium was removed by aspiration, and 10 ml of fresh serum-free DMEM/F12 medium was added to each culture. Sarcoma cell CM was collected after a 72-h incubation, and 25 mM HEPES buffer [pH 7.4] 0.5 mg/ml leupeptin, 0.7 mg/ml pepstatin, 0.8 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 0.02% NaN₃, and 0.1% BSA (Intergen, Purchase, NY) was added. Sarcoma cell CM was clarified by centrifugation. CM was frozen and stored at -80°C until use.

Endothelial Cell Growth and Migration Assay. Endothelial cell growth and migration assays were performed as described previously (32).

In Vivo Sarcoma Xenograft Models and Treatment with DC101 and/or Doxorubicin. Confluent sarcoma cell cultures were harvested by trypsinization, washed twice by centrifugation, and resuspended in HBSS (Life Technologies, Inc.). Aliquots of 2 × 10⁶ viable sarcoma cells in 0.2 ml of Matrigel

were injected s.c. into the flank region of SCID mice. Tumor volume was measured with a caliper twice weekly. The mice were killed when tumor nodules grew beyond 1.5 cm in diameter or when the mice became moribund. The tumor nodules were carefully dissected, weighed, and fixed in a 10% phosphate-buffered formaldehyde solution (Fisher Scientific, Pittsburgh, PA) or frozen at -140°C.

Animals bearing sarcoma xenografts were treated starting when the tumor diameters reach 5 mm. Each treatment group consisted of six to eight animals. DC101 (ImClone Systems, Inc., NY), a rat IgG1 mAb that reacts specifically with the extracellular region of the murine tyrosine kinase receptor Flk-1 (34), was i.p. administered at 400 µg/dose or 800 µg/dose every 3 days for a total of seven times. Doxorubicin (American Pharmaceutical Partners, INC., Los Angeles, CA) at 6 mg/kg (standard-schedule dosage) was administered every 2 weeks for two times and doxorubicin at 1.2 mg/kg (continuous low-dose schedule dosage) was administered every 3 days for a total of eight times. Combined treatment consisted of 400 µg/dose DC101 plus 1.2 mg/kg doxorubicin given once every 3 days for a total of eight times. Control animals bearing s.c. flank tumors were treated with normal rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; 800 µg/dose) or 0.9% NaCl at the same schedules. Tumor were measured twice weekly with calipers, and tumor volumes were calculated by the formula [$\pi/6 (w_1 \times w_2 \times w_3)$], where w_1 represented the largest tumor diameter and w_2 represented the smallest tumor diameter. The body weight and general physical status of the animals were recorded every 3 days.

Angiogenesis Assays in Vivo. Angiogenesis was assessed *in vivo* as described previously (32). Briefly, 380 µl of Matrigel (Becton Dickinson, Bedford, MA) and 0.1 µg of basic fibroblast growth factor (R&D Systems, Minneapolis, MN) were mixed. A total of 0.5 ml of this modified Matrigel was injected into each SCID mouse. Mice were systemically treated with control rat IgG (800 µg/dose), low-dose doxorubicin (1.2 mg/kg) alone, DC101 (400 µg/dose) alone, or combined DC101 (400 µg/dose) and low-dose doxorubicin (1.2 mg/kg) every 3 days. Twelve days after the initial injection, the Matrigel plug was removed and dissected. The Matrigel plug was fixed in 4% formaldehyde and stained with H&E to analyze infiltrating vessels in the gel.

Analysis of Microvessel Count. For an analysis of the microvessel count in human xenografts, frozen tissues were sectioned at 8-µm thickness, mounted on positively charged slides, and stained with antimouse CD31mAb (PharMingen) using the LSAB-2 staining system (DAKO). Vessel count was determined by counting the stained vessels in a field of 0.071 mm² at a final magnification of ×620. Ten fields per histological section were included in the analysis.

Preparation of Cell Lysates and Immunoprecipitate. Immunoprecipitate was performed as described previously (34). Briefly, cells at 70–80% confluence were starved in serum-free medium for 24 h and treated with or without doxorubicin or DC101 for 1 h, and then stimulated with VEGF (80 ng/ml) for 15 min. The cells were washed and lysed in IP buffer [1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA (pH 8.0), 0.2 mM sodium ortho-vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5% NP40] and the insoluble materials were removed by centrifugation. Equal amounts of protein were incubated with DC101 or control rat IgG for 1 h at 4°C and precipitated with protein A-agarose. The immunoprecipitates were washed four times with the IP buffer and eluted by boiling for 5 min in sample buffer before separation by SDS-PAGE.

Western Blot. Western blot was performed as described previously (32). Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Western blot was performed using the enhanced chemiluminescence (ECL) detection system (Amersham Corp., Arlington Heights, IL).

DNA Ladder. DNA ladder assays were performed as described previously (35). Briefly, cells growing in log-phase were washed and exposed to various agents for 6 h, and then all cells, both floating and adherent, were collected, washed in PBS, and then lysed in a buffer containing 10 mM Tris (pH 8.0), 10 mM NaCl, 100 µM EDTA (pH 8.0), and 0.5% SDS. Lysates were treated with proteinase K overnight at 56°C, followed by DNA extraction using phenol: chloroform and ethanol precipitation. Thirty µg of DNA is separated in a 1.5% agarose gel, and the gel was stained with ethidium bromide and photographed.

TUNEL Assay. To assess apoptosis, cells were plated at 5 × 10⁵/60-mm dish, allowed to adhere overnight, and then treated as described in the DNA ladder assay. Cells were trypsinized, washed with PBS, fixed in ice-cold

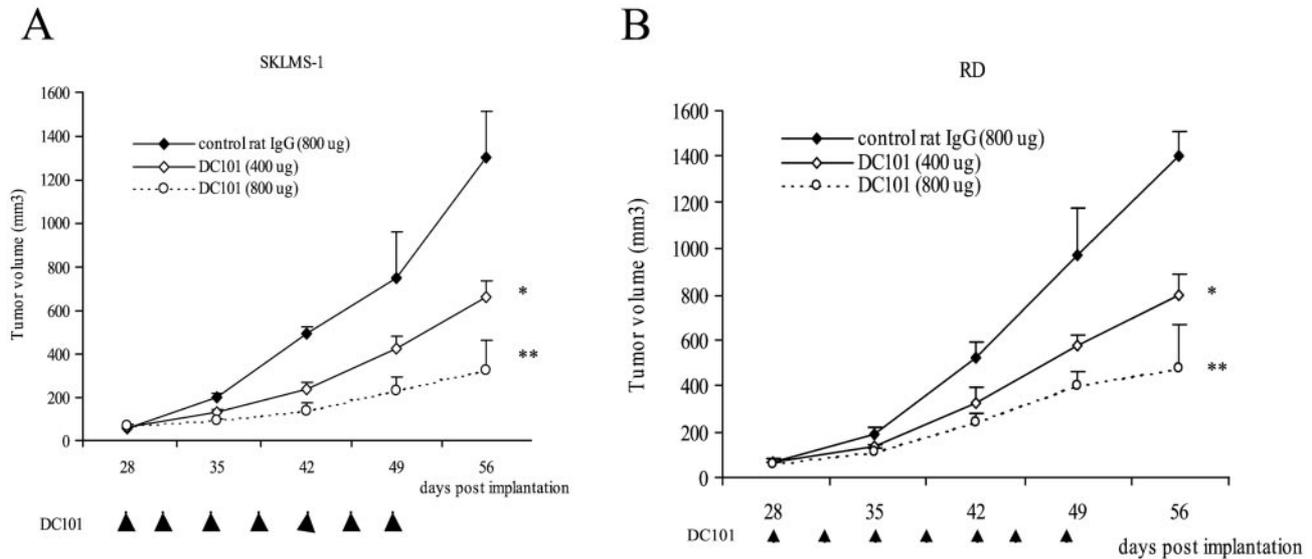


Fig. 1. Antitumor activity of DC101 against well-established SKLMS-1 (A) and RD (B) xenografts in SCID mice. Animals bearing sarcoma xenografts were treated starting when the tumor diameters reached 5 mm. DC101 as a single agent was given i.p. every 3 days for seven doses. Arrows, days on which treatment was administered. Results are given as mean tumor volume of eight mice/group. Bar, SD. *, $P < 0.05$ versus control; **, $P < 0.01$ versus control.

methanol, stored at -20°C overnight, and stained according to the manufacturer's instructions (Phoenix Flow System, Inc., San Diego, CA). Cells were analyzed by flow cytometry.

Caspase Assay. Cells were treated with various agents as indicated, rinsed with cold PBS twice, and then centrifuged and resuspended the cell pellets (6×10^5 cells/ μl) in 50 mM PIPES/KOH (pH 6.5), 2 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 5 mM DTT, 20 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ pepstatin A, 10 $\mu\text{g}/\text{ml}$ aprotinin, and 2 mM phenylmethylsulfonyl fluoride. Cells were subjected to three freeze/thaw cycles in dry ice/methanol. Lysate was centrifuged at 4°C for 30 min at $20,000 \times g$ and supernatant were recovered. Two hundreds μg of protein lysate were used for caspase 3 expression assay by Western blot (Caspase 3/Ab-1, Cat AM 20, Oncogene, Boston, MA). For the caspase 3 activity assays, a caspase-3 cellular activity assay kit (Calbiochem, San Diego, CA) was used according to the manufacturer's instructions.

Statistical Analysis. The statistical differences for microvessel counts, cell migration and proliferation, and apoptotic cell were analyzed using the Student's t test.

RESULTS

Inhibitory Effect of Anti-Flk1 mAb DC101 on Well-Established Human STS Xenografts. Initially, we set out to determine whether DC101 inhibits growth of human STS. We used 400 $\mu\text{g}/\text{dose}$ or 800 $\mu\text{g}/\text{dose}$ of DC101, according to a previous report (21), to examine the effect of DC101 on human STS xenografts in SCID mice. SCID mice bearing tumor xenografts from injection of two independent sarcoma cell lines, human leiomyosarcoma SKLMS-1 and rhabdomyosarcoma RD, were systemically treated i.p. with DC101 or control normal rat IgG every 3 days for 7 times. As shown in Fig. 1, DC101 at a dose of 800 μg significantly inhibited the growth of s.c. tumor xenografts of SKLMS-1 (Fig. 1A) and RD (Fig. 1B) compared with control groups ($P < 0.01$ for both tumor models). Complete tumor eradication was seen in three of eight mice xenografted with SKLMS-1 and two of eight mice xenografted with RD with this dose. No significant animal toxicity was observed. Because the 400- μg dose of DC101 modestly inhibited the growth of SKLMS-1 and RD xenografts, it was chosen for the subsequent combination treatment studies.

Effect of Continuous Low-Dose Doxorubicin on Growth of Well-Established Human STS Xenografts. Before examining whether continuous low-dose doxorubicin affects the growth of hu-

man STS *in vivo*, we tested the sensitivity of HUVECs, leiomyosarcoma cell line SKLMS-1, and rhabdomyosarcoma cell line RD to doxorubicin in monolayer cultures. As shown in Fig. 2A, the growth of all three cell lines was strongly inhibited at the higher concentrations of doxorubicin. However, the lowest concentration doxorubicin (*i.e.*, 0.0078 μM) inhibited endothelial cell growth but not SKLMS-1 and RD cell growth. These data suggested that endothelial cell HUVECs (as well as MluEs, data not shown) are more sensitive to doxorubicin than sarcoma cells in the low-dose range of doxorubicin used *in vitro*.

For *in vivo* treatment, a doxorubicin dose of 6 mg/kg body weight with two injections 2 weeks apart was used as the standard dosing schedule according to previous reports (36) and our preliminary testing (data not shown). A doxorubicin dose of 1.2 mg/kg body weight of eight injections once every three days was used as the continuous low-dose antiangiogenic schedule. In mice treated with standard-schedule i.p. injection of doxorubicin on day 17 and day 31 resulted in marked inhibition of tumor growth compared with the control group (Fig. 2B, SKLMS-1, Fig. 2C, RD). In the continuous low-dose schedule, doxorubicin (1.2 mg/kg) twice weekly for 4 weeks had a moderate inhibitory effect on the growth of RD xenografts (46.5% of standard-schedule treatment) but had no significant inhibitory effects on tumor growth of SKLMS-1 xenografts. The antiangiogenic-schedule doxorubicin treatment was discontinued when body weight changed.

Combined Low-Dose DC101 and Continuous Low-Dose Doxorubicin Resulted in an Enhanced Inhibitory Effect on Well-Established Human STS Xenografts. Because low-dose doxorubicin alone was not sufficient to inhibit human STS xenografts in the above experiments, we next explored whether DC101 could enhance the inhibiting effect of low-dose doxorubicin on sarcoma xenografts. A lower dose of DC101 (400 μg) was used in the combination studies with continuous low-dose doxorubicin (1.2 mg/kg) to treat well-established human STS xenografts in SCID mice. Mice were divided into five groups: control rat IgG (800 $\mu\text{g}/\text{dose}$) plus 0.9% NaCl, DC101 (800 $\mu\text{g}/\text{dose}$) plus 0.9% NaCl, DC101 (400 $\mu\text{g}/\text{dose}$) plus 0.9% NaCl, doxorubicin (1.2 mg/kg) plus control rat IgG (400 $\mu\text{g}/\text{dose}$), and doxorubicin (1.2 mg/kg) plus DC101 (400 $\mu\text{g}/\text{dose}$). As shown in Fig. 3, the efficacy of low-dose doxorubicin to inhibit

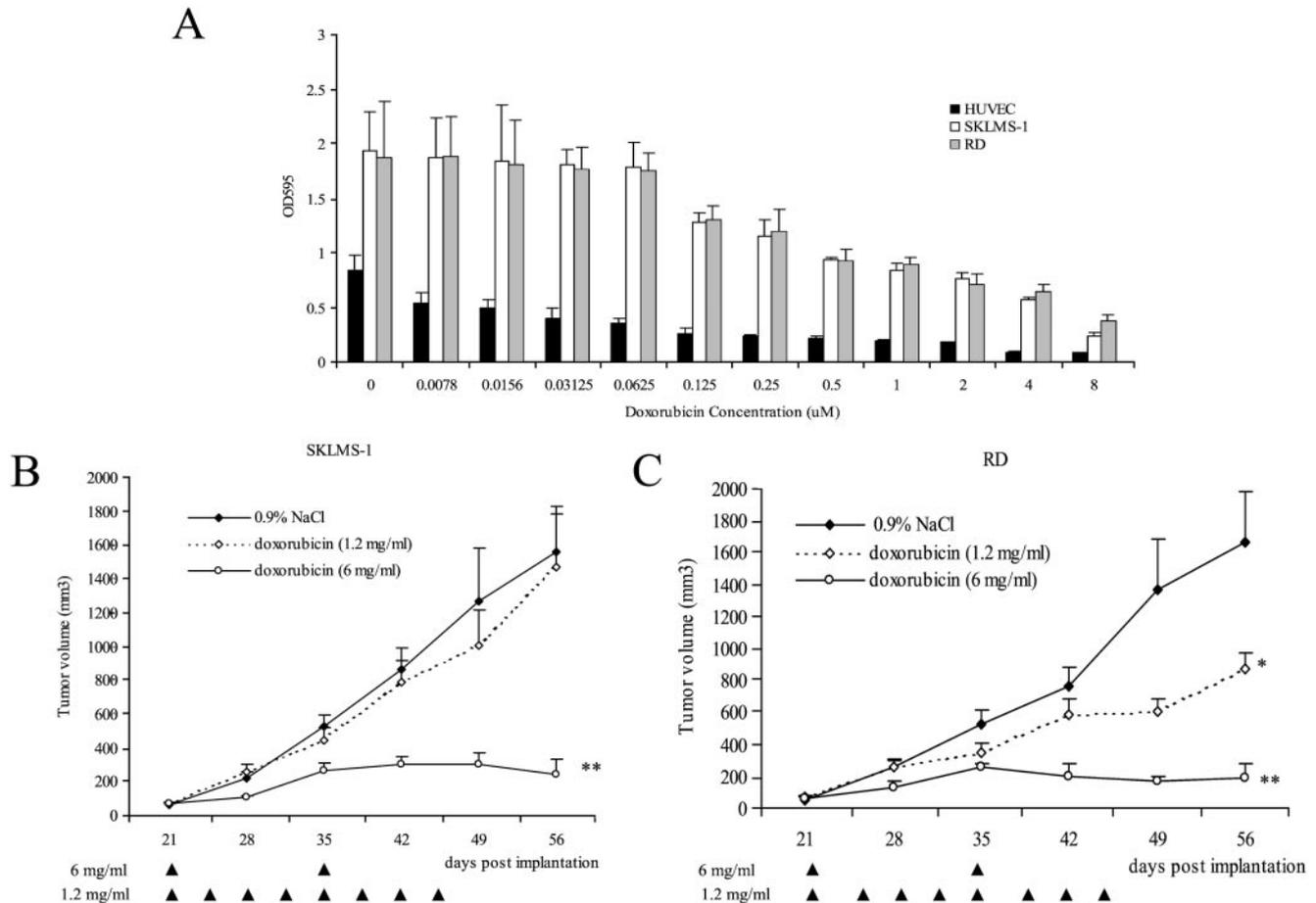


Fig. 2. Growth inhibition by continuous low-dose *versus* standard-dose scheduling of doxorubicin for SKLMS-1 and RD xenografts. Animals bearing sarcoma xenografts were treated starting when the tumor diameters reached 5 mm. Arrows, days on which treatment was administered. A, differential sensitivity to doxorubicin of HUVEC, leiomyosarcoma cell SKLMS-1, and rhabdomyosarcoma RD cells *in vitro*. Animals bearing sarcoma xenografts were treated starting when the tumor diameters reached 5 mm as described in "Materials and Methods." Arrows, days on which treatment was administered. B, SKLMS-1 xenografts. C, RD xenografts. Results are given as mean tumor volume of 8 mice/group. Bar, SD. *, $P < 0.05$ *versus* control; **, $P < 0.01$ *versus* control.

SKLMS-1 and RD xenografts was markedly enhanced in the presence of 400 μg of DC101 compared to that of any agent alone. Interestingly, growth-inhibitory effects resulting from combined treatment with low-dose DC101 (400 $\mu\text{g}/\text{dose}$) plus low-dose doxorubicin were superior to those from high-dose DC101 (800 $\mu\text{g}/\text{dose}$) alone (Fig. 3). Complete tumor eradication was seen in five of eight mice bearing SKLMS-1 xenografts and four of eight mice bearing RD xenografts treated with combined drugs. The observed tumor growth inhibition was maintained beyond 13 weeks in all of the DC101-plus-doxorubicin-treated animals. The animals in complete remission at 56 days were followed continuously for 114 days (SKLMS-1) and 106 days (RD) with no evidence of tumor recurrence. These data indicate that combined therapy resulted in an enhanced antitumor activity against SKLMS-1 and RD xenografts.

To evaluate the toxicity to the host animals by the DC101/doxorubicin combination therapy, body weight was plotted at regular intervals and considered a surrogate for the evaluation of drug toxicity in mice. No significant difference in body weights was seen between the combination therapy group and low-dose doxorubicin group. The weight curve of the DC101 group paralleled very closely that of the control group. Significant weight loss was observed in the animals treated with the standard doxorubicin treatment schedule. The low-dose doxorubicin group showed some weight loss, but the loss was significantly less than in the standard-schedule group (data not shown).

Combined Therapy Had an Additive Inhibitory Effect on Tumor Angiogenesis *in Vivo* and *in Vitro*. To determine whether inhibition of tumor angiogenesis contributed to the enhanced antitumor effects by combined therapy, xenografts from animals bearing s.c. SKLMS-1 and RD were immunostained with antimouse CD31 mAb to allow counting of microvessels in these tumor samples. Microvessel counts were significantly lower in DC101-plus-doxorubicin-treated xenografts than in tumors treated with either agent alone or control rat IgG (Fig. 4A). We further assessed the effect of DC101 plus doxorubicin on neovascularization *in vivo* by Matrigel plug assay. Matrigel plugs retrieved from mice treated with rat IgG (400 $\mu\text{g}/\text{dose}$), doxorubicin (1.2 mg/kg) alone, or DC101 (400 $\mu\text{g}/\text{dose}$) alone had extensive vascularization 12 days after implantation. In contrast, plugs retrieved from animals treated with the combined therapy of DC101 (400 $\mu\text{g}/\text{dose}$) plus doxorubicin (1.2 mg/kg) had markedly reduced neovascularization compared with plugs from animals treated with either agent alone or control rat IgG (Fig. 4B).

On the basis of reductions of tumor angiogenesis *in vivo*, we investigated whether DC101 and/or doxorubicin directly inhibited endothelial cell functions *in vitro*. Endothelial cell HUVECs (as well as MluEs) were treated with control rat IgG (10 μg), doxorubicin DC101 (10 μg), and DC101 (10 μg) plus doxorubicin (0.0625 μM) for 1 h. CM was collected from SKLMS-1 and RD sarcoma cells and added to these endothelial cells, which were assayed for migratory ability, proliferation capacity, and formation of tube-like structures.

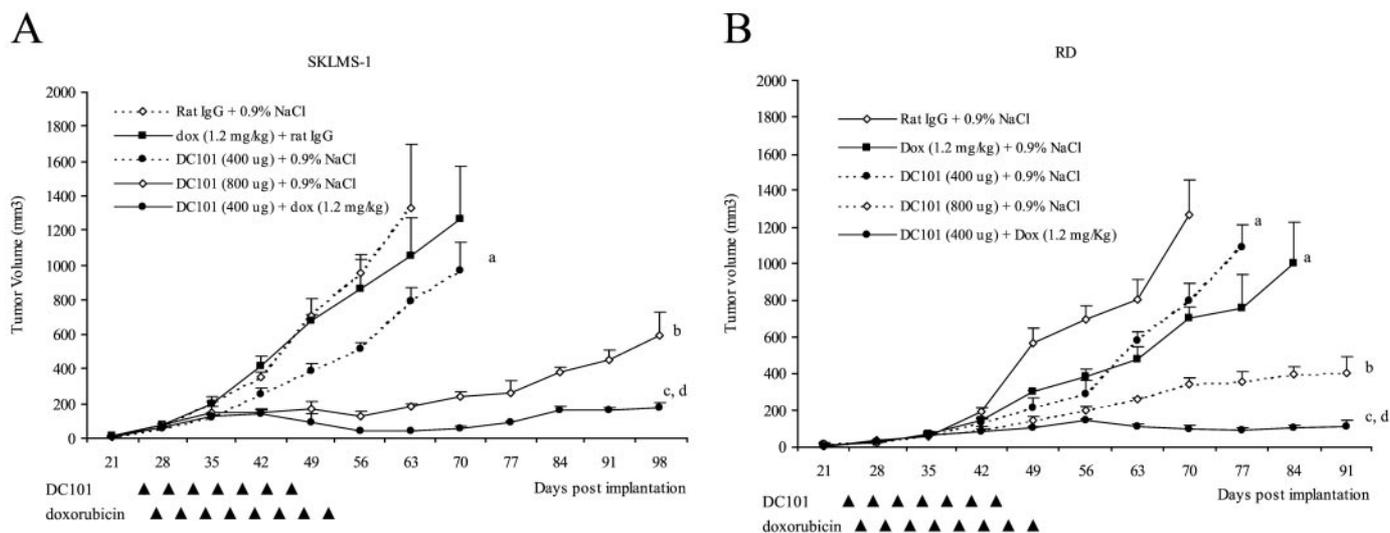


Fig. 3. Antitumor effect of combined DC101 and continuous low-dose doxorubicin against well-established human STS xenografts of SKLMS-1 (A) and RD (B). Animals bearing sarcoma xenografts were treated starting when the tumor diameters reached 5 mm as described in "Materials and Methods." Arrows, days on which treatment was administered. Results are given as mean tumor volume of 10 mice/group. Bar, SD. a, $P < 0.05$ versus control; b, $P < 0.001$ versus control; c, $P < 0.01$ versus DC101 (400 μg) alone; d, $P < 0.01$ versus continuous low-dose doxorubicin alone.

Single-agent DC101 or doxorubicin inhibited the migration and proliferation of HUVECs (as well as MluEs, data not shown; Fig. 4, C and D). The tube-like formation was also reduced by each agent (data not shown). However, the combination of DC101 plus doxorubicin exerted a significant enhanced inhibitory effect on endothelial cell migration (Fig. 4C), proliferation (Fig. 4D), and tube-like formation (data not shown). These data suggested that combined DC101 and doxorubicin resulted in an enhanced inhibitory effect on angiogenesis *in vivo* and endothelial cell activity *in vitro*.

DC101-enhanced Doxorubicin-induced Endothelial Cell Apoptosis. Because VEGF binding to VEGFR-2 induces VEGFR-2 receptor tyrosine phosphorylation and activation, we next compared the effects of the different regimens of agents on VEGF-induced VEGFR phosphorylation. HUVECs (MluEs as well) were treated with control rat IgG (10 μg), doxorubicin (0.0625 μM) alone, DC101 (10 μg) alone, or DC101 (10 μg) plus doxorubicin (0.0625 μM) for 1 h, and then stimulated with VEGF (80 ng/ml) for 15 min. The immunoprecipitation of VEGF-activated receptor with DC101 or irrelevant rat IgG were performed with 10 μg of antibody or control IgG. To detect phosphorylated receptor, blots were probed with an antiphosphotyrosine antibody. We found that DC101 reduced VEGF-induced VEGFR-2 receptor phosphorylation when the antibody was prebound to cells, but doxorubicin alone did not influence VEGF-induced receptor phosphorylation (Fig. 5A). Notably, combined treatment of DC101 and doxorubicin reduced VEGF-mediated VEGFR2 phosphorylation to levels that were nondetectable by Western blot with antiphosphotyrosine antibody (Fig. 5A, Lane 4). These results indicated that the reduction of VEGFR-2 phosphorylation by DC101 may contribute to the enhanced inhibitory effect of the combined regimen on the functions of endothelial cells.

After seeing the complete inhibition of VEGFR activation by the combined treatment of DC101 and doxorubicin, we set out to further understand the mechanism behind combined doxorubicin and DC101-induced enhanced inhibition of STS. We next examined whether treatment with combined drugs could result in more efficient induction of endothelial cell apoptosis. The endothelial cell HUVEC (as well as MluE) was treated with rat IgG (10 μg), DC101 (10 μg), or a dose of 0.0625 μM doxorubicin in the presence or absence of DC101 (10 μg) or in the presence of LY294002 (80 μM). LY294002, a

phosphatidylinositol 3-kinase (PI3K) inhibitor, is a positive control to enhance doxorubicin-induced cell apoptosis. Apoptosis was detected by DNA fragmentation and caspase-3 activity assays. DNA ladders were detected in HUVECs treated with doxorubicin alone or DC101 in combination with doxorubicin (Fig. 5B). However, similar to rat IgG, DC101 alone did not induce apoptosis of HUVECs (as well as MluEs, data not shown), because no DNA ladders were detected (Fig. 5B). Flow cytometric analysis showed that the combination of doxorubicin and DC101 produced 35% apoptosis, doxorubicin alone produced 18.6% apoptosis, whereas apoptosis occurred in only 3.5% of the cells treated with DC101 alone or 3.2% with rat IgG alone (Fig. 5C). These data suggested that DC101 plus doxorubicin resulted in robust apoptotic response in HUVECs compared to doxorubicin alone.

We then investigated whether doxorubicin, or DC101 alone or in combination induced caspase-3 activity, an early marker of apoptosis, in HUVECs (as well as MluEs). Cells were treated for 6 h with rat IgG (10 μg), DC101 (10 μg), or doxorubicin (0.0625 μM) with or without DC101 (5 or 10 μg), and were harvested for Western blot analysis of caspase 3 activities. Active caspase-3 was only observed after treatment with doxorubicin alone or with the combination of DC101 (Fig. 5D). Relative caspase-3 activities were measured in cell extracts, which showed a 5.8-fold increase in caspase-3 activity when cells were treated with a combination of doxorubicin and DC101 and a 2.9-fold increase with doxorubicin alone; little caspase activation was seen with DC101 or rat IgG alone (Fig. 5E). These data suggested that DC101 facilitated caspase 3 activation induced by doxorubicin.

DISCUSSION

Our data in this study demonstrated that mAb DC101, which targets the VEGFR-2, as a single agent, has antitumor activity against human STS *in vivo*. The data also showed that the combined treatment with low-dose DC101 and continuous low-dose doxorubicin led to an enhanced antiangiogenic effect and an additive antitumor growth compared with doxorubicin or DC101 alone. To our knowledge, this is the first time that DC101 and continuous low-dose doxorubicin have been tested together and found to have additive inhibitory effects on human STS xenografts in SCID mice.

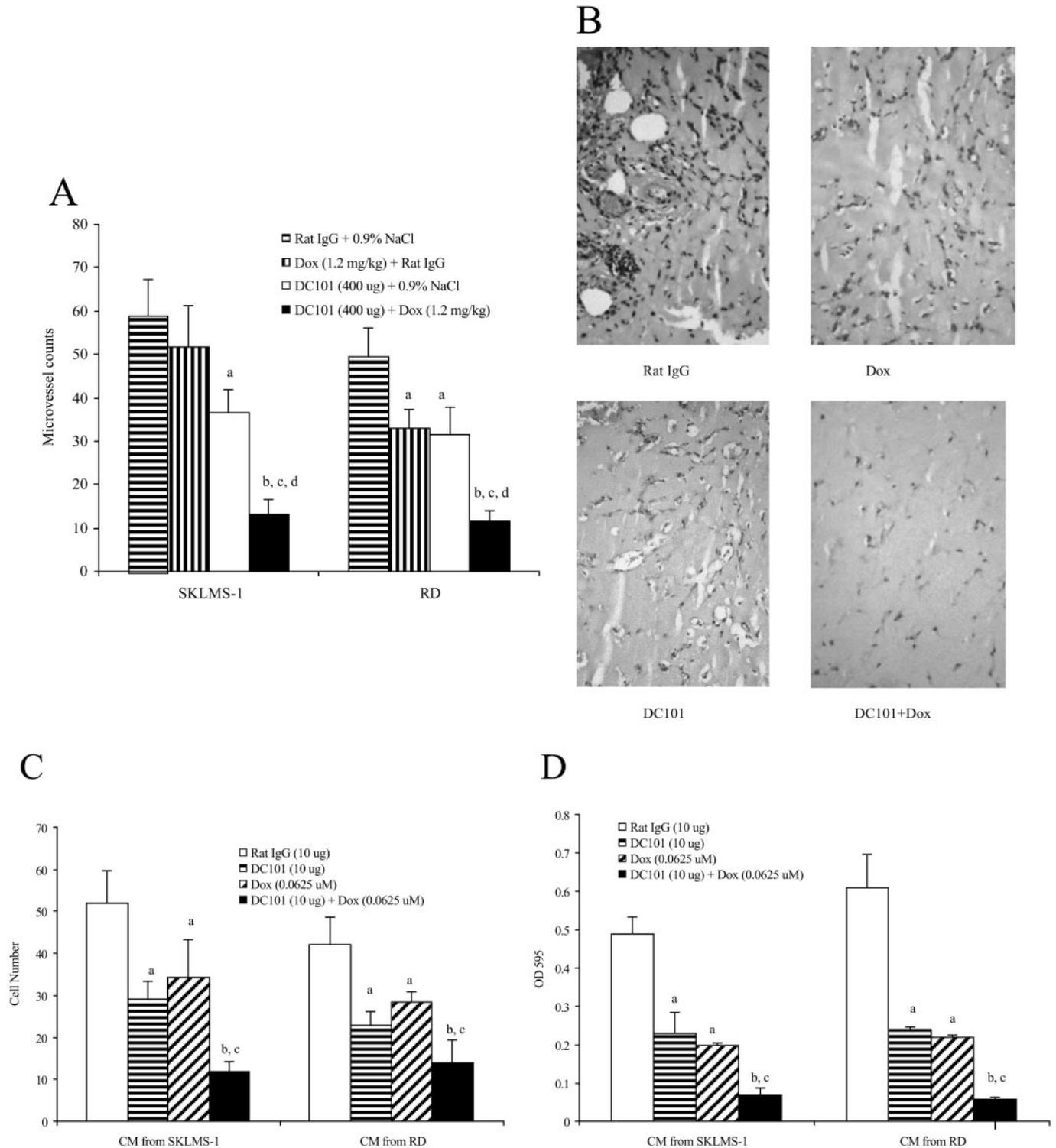


Fig. 4. DC101 plus doxorubicin enhanced the inhibition of tumor angiogenesis *in vivo* and endothelial cell activity *in vitro*. **A**, microvessel count in xenografts of various treatment group. *a*, $P < 0.05$ relative to control group; *b*, $P < 0.001$ relative to control group; *c*, $P < 0.01$ relative to continuous low-dose doxorubicin-treated-alone group; *d*, $P < 0.01$ relative to DC101-treated-alone group. **B**, histology of recovered Matrigel plug from variously treated mice. Representative fields showed enhanced inhibition of neovascularizations by DC101 plus doxorubicin compared with either agent alone or control rat IgG. **C** and **D**, endothelial cell migration (**C**) and growth (**D**) assay. Cells were variously treated as indicated. CM from SKLMS-1 or RD cells was added to these treated cells, and the assay was performed as described previously (32). *a*, $P < 0.01$ relative to control group; *b*, $P < 0.001$ relative to control group; *c*, $P < 0.05$ relative to either agent alone. Bars, SD.

Inhibition of VEGF/VEGFR-mediated tumor angiogenesis, *e.g.*, through VEGFR tyrosine kinase inhibitor (20), soluble VEGFRs (19), antisense-*VEGF* nucleotide (17), or humanized neutralizing anti-VEGF antibody (18), were shown to prevent tumor growth and metastasis. Previous studies have shown that anti-Flk1 mAb inhibited

tumor growth in several other types of tumor xenografts (21, 37–41). Human STSs express a number of proangiogenic factors, *e.g.*, VEGF/VEGFR, that may represent therapeutic targets. In the present study, we targeted VEGFR2 activation by the anti-Flk1 mAb DC101 as a therapeutic strategy against human STS xenografts in SCID mice.

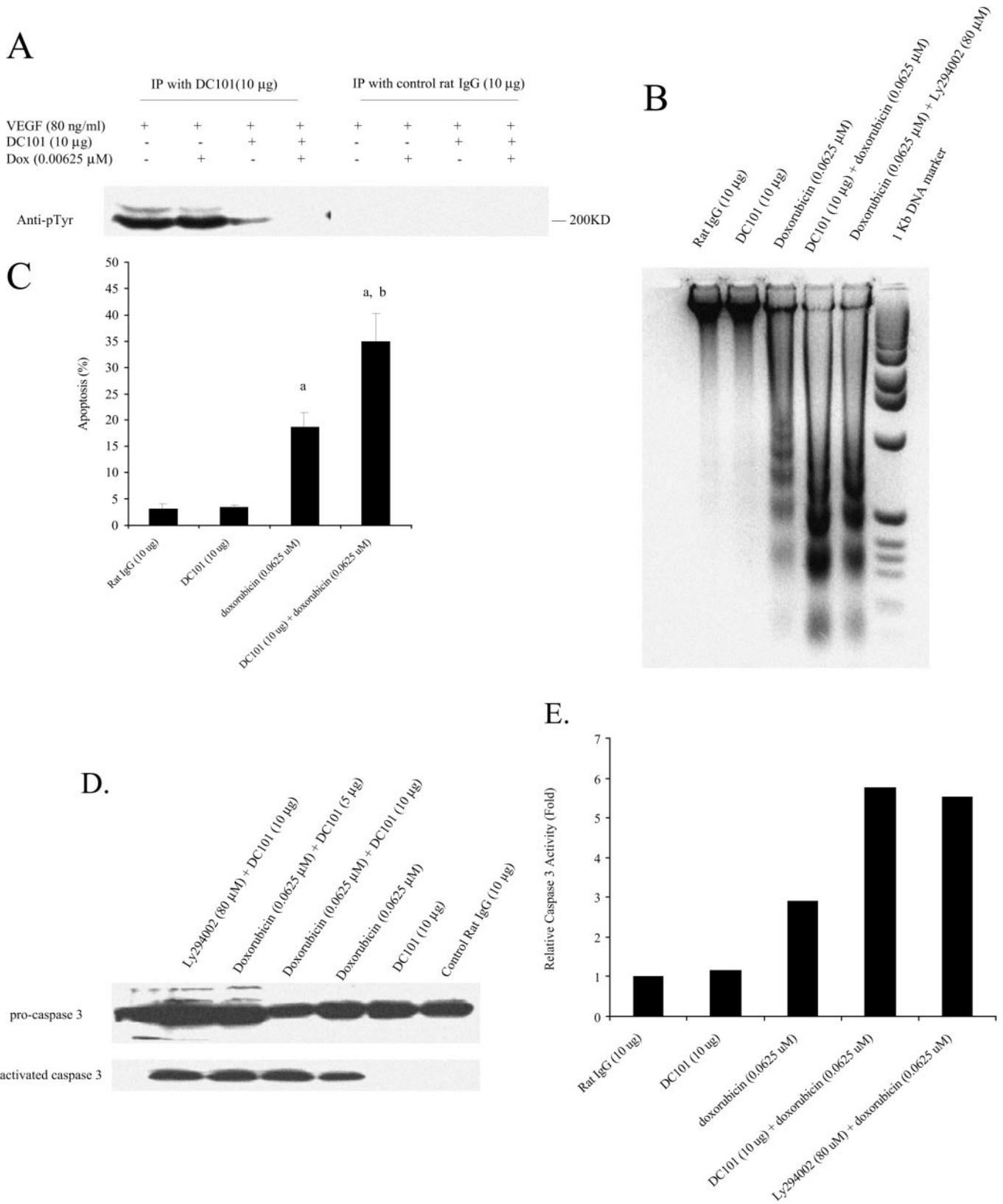


Fig. 5. DC101-enhanced doxorubicin-induced endothelial cell apoptosis. *A*, DC101 reduced VEGF-induced Flk-1 phosphorylation. Cell lysates containing equal amounts of proteins were immunoprecipitated with DC101 or control rat IgG. Immunoprecipitates were separated on 8% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were hybridized with antibody against phosphotyrosine. *B*, DNA fragmentation assay. Genome DNA was prepared as described in "Materials and Methods." Aliquots of 30 μ g of DNA were loaded in each lane, separated by agarose gel electrophoresis, and visualized by ethidium bromide staining. LY 294002 + doxorubicin as a positive control. *C*, TUNEL assay. Cells were prepared as described in "Materials and Methods" and analyzed by flow cytometry. *a*, $P < 0.001$ relative to control; *b*, $P < 0.001$ relative to either agent alone. *D*, Western blot assay for active caspase-3. Cell lysates were prepared as described in "Materials and Methods." An aliquot of 200 μ g of protein was loaded in each lane. The active form of caspase-3 was detected using a mAb specific for the active form of caspase-3 (PharMingen). The precursor (inactive) form of caspase-3 was detected using a mAb specific for the unprocessed protein (Oncogene). *E*, relative caspase-3 activity assay. Relative caspase-3 activities were assayed using caspase-3 cellular activity kit (Calbiochem). Relative activity is reported compared with control cells. Columns (fold), averages of duplicate caspase measurements.

Treatment with DC101 alone significantly retarded the growth of SKLMS-1 and RD xenografts in SCID mice and inhibited tumor angiogenesis *in vivo*. These results provide evidence that antiangiogenic therapy, mediated by blocking the VEGF/VEGFR-pathway, can inhibit human STS growth *in vivo*.

The concept of antiangiogenic-schedule chemotherapy was initially proposed to target the endothelial cells, which can recover during the rest period and restore the tumor's blood supply during standard intermittent chemotherapy, and to give drugs at lower doses and more frequent intervals without overt toxicity (24, 25). This new strategy is currently being explored as an antitumor experimental therapy (22–25). Because doxorubicin is the most active chemotherapeutic agent against human STS with the highest response rate of about 25% (29), we conducted a series of animal experiments with continuous low-dose and standard-dose doxorubicin and tested whether the low-dose schedule would induce an antiangiogenic effects *in vivo* with fewer side effects. Our data showed that standard-dose treatment resulted in the inhibition of tumor growth (Fig. 2) as well as significant toxicity as represented by weight loss (data not shown). Treatment with the antiangiogenic schedule inhibited tumors in RD xenografts but not in SKLMS-1. These results suggested that the efficacy of antiangiogenic schedule “metronomic” chemotherapy may be dependent on the type of sarcoma. The low-dose doxorubicin group showed some weight loss, but it was significantly lower than the standard schedule group. This is consistent with a recent publication suggesting that antiangiogenic activity requires different drug doses and schedules than those required for optimal cytotoxicity (22). Additional studies to elucidate the antiangiogenic doses and schedule of doxorubicin in different human STS sarcoma xenograft models are on going in our laboratory.

It should be emphasized that DC101-treated animals eventually succumbed to tumor burden in our experiments. A previous report also showed that withdrawal of DC101 treatment in the various models resulted in regrowth of tumors with kinetics similar to those of control groups (21). These studies further support the notion that antiangiogenic treatment needs to be combined with other treatment modalities, *e.g.*, chemotherapy or radiation therapy. Several reports showed that DC101 used in combination with chemotherapeutic agents, *e.g.*, paclitaxel or vinblastine, and radiotherapy led to markedly enhanced antitumor activity in other tumor types (38). In our study, the combined treatment with lower-dose DC101 and continuous low-dose doxorubicin led to an enhanced antitumor effect compared with doxorubicin or DC101 alone. These results indicate that DC101 enhances the antitumor activity of continuous low-dose doxorubicin in human STS xenografts as well. In addition, we also found that combined therapy has an additive inhibitory effect on angiogenic response as compared with DC101 alone.

The mechanisms responsible for the interaction between mAb DC101 and doxorubicin are not fully understood but likely involve an increased apoptosis of the endothelial cell population coupled with decreased proliferation of endothelial cells. The enhanced apoptosis represents the summation of effects on multiple pathways regulating apoptosis. Doxorubicin has a significant antitumor effect for human STS, either as a single agent or in combination with other cytotoxic agents. Doxorubicin led to DNA breakage and eventually resulted in cell apoptosis in many tumor cells. Our results show that doxorubicin can also induce endothelial cell apoptosis. VEGF is a survival factor for endothelial cells by inducing antiapoptotic signaling pathways (42). In the present study, treatment with DC101 plus doxorubicin led to significant reductions in microvessel counts in xenografts as compared with treatment with any agent alone. DC101 plus doxorubicin inhibited endothelial cell functions *in vivo*, as supported by data from *in vitro* assays. We observed that DC101 alone blocked VEGFR-2 activation and led directly to the inhibition of endothelial cell migra-

tion, proliferation, and tube-like formation. Moreover, DC101 plus doxorubicin had an additive inhibitory effect on these endothelial cell functions. Together, the inhibitory effects of DC101 plus doxorubicin on tumor angiogenesis in human STS xenografts *in vivo* coincide with the *in vitro* data, confirming the inhibitory effects on endothelial cell functions *in vitro*. Thus, antiangiogenic therapy using anti-Flk1 antibody DC101 plus continuous low-dose doxorubicin inhibited human STS xenograft growth, mostly because of the inhibition of tumor angiogenesis resulting from direct inhibitory effects on endothelial cell functions and induction of endothelial cell apoptosis. The results of the present preclinical study may serve as a basis for future clinical trials using this new strategy of DC101 plus continuous low-dose doxorubicin combination therapy for treatment of human STSs.

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Combined Anti-Fetal Liver Kinase 1 Monoclonal Antibody and Continuous Low-Dose Doxorubicin Inhibits Angiogenesis and Growth of Human Soft Tissue Sarcoma Xenografts by Induction of Endothelial Cell Apoptosis

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