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Inhibition of Tumor-induced Angiogenesis by a Synthetic Lipid A Analogue with Low Endotoxicity, DT-5461

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We investigated the effect of a synthetic lipid A analogue with low endotoxicity, DT-5461, on the neovascularization induced by B16-BL6 melanoma in syngeneic mice. A systemic single administration of DT-5461 caused a marked decrease in the number of vessels oriented toward the tumor mass and in the tumor size during the early phase of vasculogenesis (on day 4 after tumor inoculation), with little or no inhibition in the following phases. Multiple i.v. administrations of DT-5461 at intervals of 4 days (an effective schedule for inhibiting tumor metastasis) significantly reduced the number of capillary vessels and tumor growth over a period of 14 days after the tumor implantation. Multiple systemic administrations of DT-5461 on days 1, 5 and 9 after tumor inoculation caused a high production of endogenous tumor necrosis factor-α (TNF-α) in tumor sites although this treatment modality induced a low production in serum of tumor-bearing mice. Tumor homogenate from mice treated with DT-5461 suppressed the proliferation of endothelium *in vitro*, whereas sera from animals given DT-5461 had little effect. Furthermore, the antiproliferative effect of the tumor homogenate from mice treated with DT-5461, was completely abrogated by anti-mTNF-α monoclonal antibody (mAb). The anti-angiogenic effect of DT-5461 was also completely abrogated by rabbit anti-mouse tumor necrosis factor-α (anti-mTNF-α) antiserum, whereas the inhibition of tumor growth by DT-5461 was only slightly diminished. Tumor homogenate from mice treated with DT-5461 suppressed the proliferation of endothelium *in vitro*, whereas sera from animals given DT-5461 had little effect. Furthermore, the antiproliferative effect of the tumor homogenate from mice treated with DT-5461 was completely neutralized by anti-mTNF-α mAb. Multiple i.v. administrations of DT-5461 after s.c. implantation of B16-BL6 cells significantly inhibited the growth of primary tumors measured at the time of tumor excision on day 21, and the lung metastasis of melanoma cells as compared with the untreated control in the spontaneous metastasis model. These results suggested that the suppressive effect upon tumor-associated angiogenesis by DT-5461 contributes in part to the inhibition of tumor metastasis.

Key words: Synthetic lipid A analogue — TNF-α — Melanoma — Tumor-induced angiogenesis — Lung metastasis

Angiogenesis, the development of a vascular formation, is a crucial event in various physiological and pathological states.[1-3] The process of tumor-associated angiogenesis involves several sequential steps, including degradation of the basement membrane of the parent vessels, migration and invasion of endothelial cells toward the tumor site, endothelium proliferation and formation of a capillary sprout.[4] Solid tumors require the development of a vascular network for their progressive growth.[4] Angiogenesis also allows tumor cells to metastasize from primary or secondary sites to distant organs.[5] Inhibition of neovascularization might therefore be an effective means of suppressing tumor growth and metastasis.

Previous studies have demonstrated that several agents, such as protamine,[6] platelet factor 4,[7] tumor necrosis factor-α (TNF-α),[8] interferon-α (IFN-α),[9] and bacterial cell wall complex,[10] are effective for the inhibition of tumor angiogenesis. We previously reported that a polypeptide containing the Arg-Gly-Asp (RGD) sequence of fibronectin, poly(RGD), and a sulfated chitin derivative, SCM-chitin, neither of which directly affected the viability or growth of the endothelial cells, inhibited both tumor-induced angiogenesis in syngeneic mice and the invasion of endothelial cells through the reconstituted basement membrane *in vitro*, resulting in the inhibition of experimental or spontaneous metastasis of B16-BL6 melanoma cells.[11-14]

Lipopolysaccharide (LPS), an endotoxin of gram-negative bacteria, reportedly possesses various biological activities, and lipid A has been shown to be an active

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moiety responsible for the biological action of LPS.[15] It has been reported that the administration of LPS results in the suppression of the progressive growth of solid tumors in mice, and TNF-α is a responsible for mediating the LPS-induced hemorrhagic necrosis and regression of established tumors.[16, 17] However, LPS is known to cause severe side effects, including lethal toxicity or pyrogenicity, and is unsuitable for the treatment of cancer. Previous studies have shown that DT-5461, a synthetic lipid A analogue with low endotoxicity, possesses antitumor effects against various murine syngeneic solid tumors.[18, 19] We reported that DT-5461 significantly inhibited liver metastasis of L5178Y-ML25 lymphoma cells and lung metastasis of B16-BL6 melanoma cells in experimental and spontaneous metastasis models in mice.[20] Furthermore, multiple administrations of DT-5461 remarkably prolonged the survival of L5178Y-ML25 lymphoma- or B16-BL6 melanoma-bearing mice as compared with untreated controls.[20]

In this study, we investigated the effect of DT-5461 on the neovascularization caused by solid tumors in syngeneic mice, to clarify the mechanism responsible for the antimetastatic effect of DT-5461.

MATERIALS AND METHODS

Animals Specific pathogen-free female C57BL/6 mice, 7–10 weeks old, were purchased from the Shizuoka Laboratory Animal Center, Hamamatsu. The animals were maintained in the Laboratory of Animal Experiment, the Institute of Immunological Science, Hokkaido University, under laminar air-flow conditions.

Cells and cell cultures The highly metastatic subline of murine B16 melanoma, B16-BL6, was provided by Dr. I. J. Fidler, M.D. Anderson Cancer Center, Houston, TX, and a TNF-α-sensitive fibroblast cell line originally derived from mice of the C3H strain, L929, was provided by Chugai Pharmaceutical Co., Ltd., Tokyo. The cells were maintained as monolayer cultures in Eagle's minimal essential medium (EMEM) supplemented with 7.5% fetal bovine serum (FBS), vitamins, sodium pyruvate, nonessential amino acids, and L-glutamine. Rat lung endothelial (RLE) cells were provided by Dr. M. Nakajima, Institute of Molecular and Cellular Bioscience, Tokyo University, Tokyo, and maintained in 1.0% gelatin-coated plastic tissue culture plates containing Dulbecco's modified Eagle's medium and Ham's F-12 (DMEM:F-12) supplemented with 7.5% FBS.

Reagents DT-5461 was kindly provided by Daiichi Pharmaceutical Co., Ltd., Tokyo and synthesized as described previously.[18] LPS (*E. coli* 0127:B8) was purchased from Difco Laboratories (Detroit, MI). DT-5461 and LPS were solubilized in 1 mg/ml of *N*-methyl-D-glucamine (meglumine)-5% glucose before use. Recombinant mouse TNF-α (rmTNF-α) and rabbit anti-mTNF-α antiserum were purchased from Genzyme (Cambridge, MA). Rat anti-mTNF-α monoclonal antibody (mAb) was purchased from UBI, Inc. (New York, NY).

Assay for tumor-induced angiogenesis Tumor angiogenesis in syngeneic mice was assayed as described previously[21] with some modifications.[14] C57BL/6 mice were inoculated intradermally (i.d.) with B16-BL6 melanoma cells (5×10^5) at two sites on the back. Mice were given an intravenous (i.v.) or intratumoral (i.t.) injection of various doses of LPS (50 μg/head), DT-5461 (10, 50, 250 μg/head) or rmTNF-α (50 ng/head) with or without simultaneous i.t. injection of rabbit anti-mTNF-α antiserum (50 μl/head) on various days after tumor inoculation. Three days after the administration, the mice were killed immediately after an i.v. injection (0.2 ml) of 1% Evan's blue, and the skins were separated from the underlying tissues. The inoculation sites were located with a dissecting microscope, and angiogenesis was quantified by counting the number of vessels oriented toward the tumor mass. The tumor size was estimated by averaging the short and long axes of the remnant of injected cells. All measurements were made by a single observer in a blinded manner.

Preparation of serum and tumor homogenate from tumor-bearing mice Five C57BL/6 mice per group were inoculated i.d. with B16-BL6 melanoma cells (5×10^5) at two sites on the back and the multiple i.v. administrations of 50 μg/head of LPS or DT-5461 were given on day 1, 5 or 9 after the tumor inoculation. Mice were bled 2 h after the last injection, then the sera were pooled and stored at -20°C until use. The tumor masses were then obtained and homogenized in Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (5 ml/g tumor mass) on ice. The cell-free supernatants of tumor homogenates were obtained by centrifugation at 800*g* for 30 min and kept at -20°C before use.

TNF-α assay TNF-α activity was measured by a cytotoxicity assay with actinomycin D-treated L929 fibroblasts as described previously.[22] Briefly, L929 cells (5×10^3/well) suspended in EMEM supplemented with 2.5% FBS were seeded into a 96-well plate (Costar, Cambridge, MA). Several hours later, various dilutions of sera or tumor homogenates, or serial two-fold dilutions of standard rmTNF-α were added to the well, followed by the addition of actinomycin D at a final concentration of 0.05 μg/ml. The cultures were incubated at 37°C for 48 h, washed with tap water and stained with 0.5% crystal violet in 20% methanol for 15 min. After washing, the residual cells were solubilized with 0.1 ml of 30% acetic acid. The absorbance at 590 nm was monitored photometrically with an MTP-22 dual-wavelength microplate photometer (Corona Electric Co., Ltd., Tokyo). The value for TNF-α activity was expressed as pg/ml

calculated from a standard curve in triplicate cultures. The lower and upper limits of sensitivity of the assay are 0.1 ng/ml and 100 ng/ml, respectively.

Cell proliferation assay RLE cells suspended in DMEM: F12 containing 7.5% FBS were seeded at 3×10^3 cells/well in a 1% gelatin-coated 96-well tissue culture plate (Becton Dickinson, Lincoln Park, NJ) and were allowed to attach for 4 h. The cultures were then incubated with or without 40-fold dilutions of sera or tumor homogenates, or rmTNF-α (100 ng/ml) at 37°C for 48 h. In another experiment, tumor homogenates or standard rmTNF-α were incubated with control IgG or anti-mTNF-α mAb (1 μg/ml) at 37°C for 30 min before being added to the cultures. The cultures were pulsed with 0.5 μCi/well of [^{3}H]thymidine (specific activity, 23 Ci/mmol; Amersham International, Buckinghamshire) for the final 4 h. The cells were then harvested using Filtermate 196 (Packard Instrument Company, Meriden, CT) and the radioactivity was measured in a Matrix 96 direct beta counter (Packard Instrument Company). The radioactivity was expressed as counts per minute, mean$\pm$ standard deviation (SD) in quadruplicate cultures.

Assay for spontaneous lung metastasis of melanoma cells Eight C57BL/6 mice per group were injected s.c. with 5×10^5 B16-BL6 melanoma cells into the right hind footpad and the primary tumors that formed were surgically removed by amputation on day 21. Multiple injections of DT-5461 (50 μg/head) were given the day after tumor inoculation and every 4 days thereafter for 35 days. The mice were killed 35 days after the tumor inoculation and the lungs were fixed in Bouin's solution. The lung tumor colonies were counted under a dissecting microscope.

Statistical analysis The statistical significance of differences between groups was determined by applying Student's two-tailed t test.

RESULTS

Inhibition of tumor neovascularization by DT-5461 Neovascularization induced by solid tumors is considered to be an important event in progressive growth and metastasis from primary and secondary sites to distant organs.[5] We previously demonstrated that multiple injections of DT-5461 markedly reduced the number of lung metastatic colonies of melanoma cells and the size of the primary tumor in a spontaneous metastasis model in mice.[20] We investigated the effect of DT-5461 on tumor-induced angiogenesis in syngeneic mice to analyze the mechanism of the antimetastatic effect. Mice were given an i.v. injection of LPS or DT-5461 the day after an i.d. inoculation of B16-BL6 cells. Tumor size and the angiogenic response (the number of vessels oriented toward the tumor mass) were measured 4 days after the tumor inoculation under a dissecting microscope (Fig. 1). The single administration of LPS significantly inhibited the angiogenic response and tumor mass growth compared with untreated controls (Table I). The single administration of DT-5461 also markedly decreased the number of

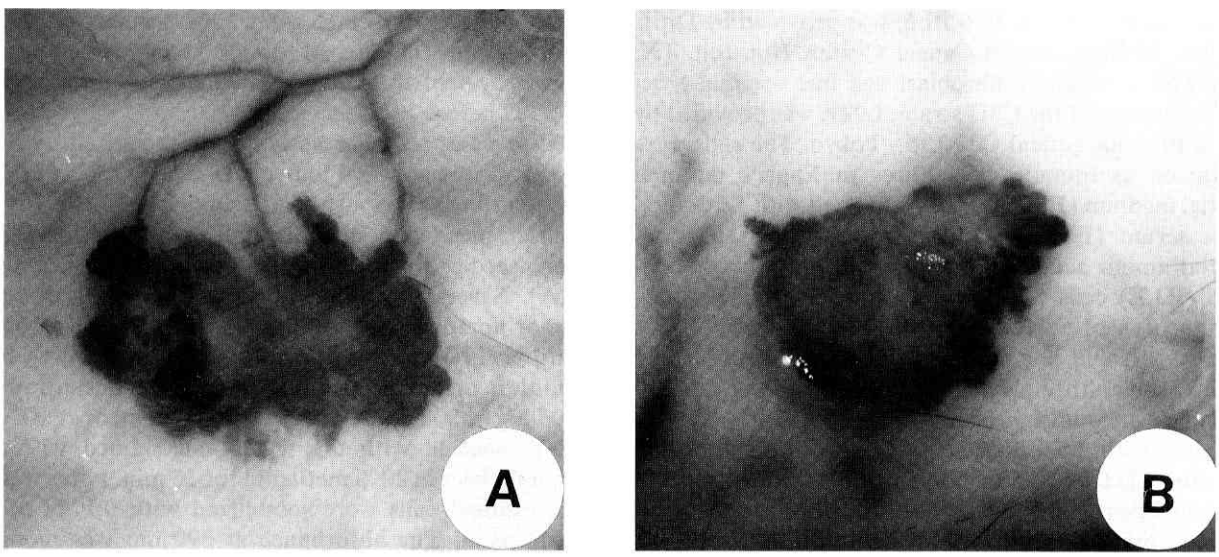

Fig. 1. Photomicrographs of tumor-induced angiogenesis. Mice were injected i.v. with (B) or without (A) DT-5461 (50 μg/head) after an i.d. inoculation of B16-BL6 melanoma cells (5×10^5). Three days after the injection, mice were killed immediately after i.v. injection (0.2 ml) of 0.1% Evan's blue and the skin was separated from the underlying tissue.

Table I. Inhibitory Effect of DT-5461 on Tumor-induced Angiogenesis

Administered i.v. with:	Dose (μg/mouse)	Angiogenesis (mean no. of vessels±SD)	Tumor size (mm)
Untreated (PBS)	—	22±6	4.2±0.4
LPS	50	12±1[b]	3.2±0.6[a]
DT-5461	10	16±3[a]	3.8±0.4
	50	12±4[b]	3.3±0.4[b]
	250	13±3[b]	3.6±0.2[b]

Three C57BL/6 mice per group were given an i.v. injection of the indicated doses of LPS or DT-5461 the day after an i.d. inoculation of B16-BL6 cells (5×10^5) at two sites on the back. Three days after the injection of LPS or DT-5461, the mice were killed and the skin was separated from the underlying tissues. Angiogenesis was quantified by counting the number of vessels oriented toward the tumor mass.
a) $P<0.05$, *b*) $P<0.01$ as compared with the untreated control.

Table II. Effect of Multiple Administrations of DT-5461 on Tumor-induced Angiogenesis

Administered i.v. with:	Evaluation (on Day)	Angiogenesis (mean no. of vessels±SD)	Tumor size (mm)
Day 1			
Untreated (PBS)	4	12±3	4.3±0.4
DT-5461	4	8±2[a]	3.1±0.4[b]
Day 1, 5			
Untreated (PBS)	8	18±2	7.2±1.1
DT-5461	8	12±3[b]	5.1±0.9[b]
Day 1, 5, 9			
Untreated (PBS)	12	22±2	10.3±0.6
DT-5461	12	17±3[b]	8.8±1.0[b]

Three C57BL/6 mice per group were given i.v. injections of 50 μg/head of DT-5461 on the indicated days after an i.d. inoculation of B16-BL6 cells (5×10^5) at two sites on the back. Three days after the last injection, the mice were killed and the skin was separated from the underlying tissues. Angiogenesis was quantified by counting the number of vessels oriented toward the tumor mass and tumor size was measured.
a) $P<0.05$, *b*) $P<0.01$ as compared with the untreated control.

vessels oriented toward the tumor mass and the tumor size in the dose range from 10 to 250 μg/head (Table I).

We next examined the effect of DT-5461 upon neovascularization at various days after tumor inoculation. The single administration of DT-5461 was given on day 1, 4 or 7 after tumor implantation, and the angiogenic response was assessed 3 days after the last injection. However, the single administration of DT-5461 on day 4 or 7 did not significantly affect the number of new capillary vessels, although DT-5461 given on day 1 or 4 inhibited the tumor mass growth (data not shown). We reported that multiple intermittent administrations of DT-5461 at intervals of 4 days inhibited tumor metastasis.[20] We therefore examined the effect of multiple administrations of DT-5461 on tumor-induced angiogenesis. Mice were injected 2 or 3 times with DT-5461 at 4-day intervals, then the tumor size and the number of vessels oriented toward the tumor mass were measured 3 days after the last injection. Table II shows that both schedules of multiple DT-5461 administration were effective in suppressing tumor-induced angiogenesis and tumor growth.

Table III. Endogenous Production of TNF-α Induced by DT-5461

Administered i.v. with:	pg/ml (mean±SD)	
	Serum	Tumor mass
Untreated (PBS)	<100	220±10
LPS	1200±80	4300±170
DT-5461	280±20	2600±210

Five C57BL/6 mice per group were given i.v. injections of 50 μg/head of LPS or DT-5461 on days 1, 5 and 9 after an i.d. inoculation of B16-BL6 cells (5×10^5) at two sites on the back, and sera and tumor mass were obtained 2 h after the last injection. The concentrations of TNF-α in sera and tumor mass homogenates were determined by cell proliferation assay.

Endogenous production of TNF-α in tumor-bearing mice treated with DT-5461 We examined the effect of DT-5461 on the production of endogenous TNF-α in tumor-bearing mice. The animals were inoculated i.d. with 5×10^5 B16-BL6 cells at two sites on the back, and three intermittent injections of LPS or DT-5461 were given 1, 5 and 9 days thereafter. The systemic injections of LPS induced high endogenous serum TNF-α in mice (Table III). In contrast, there was low production in serum of mice treated with DT-5461 (Table III). The local production of TNF-α in tumor lesions is considered to be important in suppressing the progressive growth of solid tumors.[23] We observed that three intermittent administrations of DT-5461 as well as LPS significantly reduced the size and weight of the tumor mass 9 days after the tumor inoculation as compared with untreated controls (data not shown). Therefore, we examined whether multiple injections of DT-5461 can induce endogenous TNF-α in the tumor mass (Table III). Multiple i.v. injections of DT-5461 caused a high production of endogenous TNF-α in the tumor mass as compared with untreated control. On the other hand, similar results were observed in tumor-bearing mice given injections of

LPS (Table III). These results indicated that multiple injections of DT-5461 induce the production of endogenous TNF-α even in the tumor mass.

Table IV. Effect of Endogenous TNF-α Induced by DT-5461 on Endothelial Cell Proliferation

Treatment	[^{3}H]TdR uptake into RLE cells (cpm±SD)
Control culture	29285±4822
Sera	
Untreated (PBS)	32440±4023
DT-5461	25019±3474 (14%)
Tumor homogenates	
Untreated (PBS)	26163±934 (11%)
DT-5461	11057±1467 (62%)[a]
rmTNF-α (100 ng/ml)	8239±2967 (72%)[a]

Five C57BL/6 mice per group were given i.v. injections of 50 μg/head of DT-5461 on days 1, 5 and 9 after an i.d. inoculation of B16-BL6 cells (5×10^5) at two sites on the back. Sera and the tumor mass were obtained 2 h after the last injection of DT-5461. Cell proliferation was assayed in terms of [^{3}H]-thymidine uptake. rmTNF-α was used as a positive control. The values in parentheses represent % inhibition compared with the control culture.
a) $P<0.01$ as compared with the untreated control.

Table V. Abrogation of Anti-mTNF-α mAb on the Antiproliferative Effect of Endogenous TNF-α in the Tumor Mass from DT-5461-treated Mice

Treatment	[^{3}H]TdR uptake into RLE cells (cpm±SD)
Control culture	5359±591
+control IgG	6404±889
+anti-mTNF-α mAb	5056±1104 (6%)
Tumor homogenates	
Untreated (PBS)	6579±1470
DT-5461	2261±564 (56%)[a]
+control IgG	2533±369 (53%)[a]
+anti-mTNF-α mAb	5728±1224
rmTNF-α (100 ng/ml)	1854±705 (65%)[a]
+control IgG	1596±744 (70%)[a]
+anti-mTNF-α mAb	5030±266 (6%)

Five C57BL/6 mice per group were given i.v. injections of 50 μg/head of DT-5461 on days 1, 5 and 9 after an i.d. inoculation of B16-BL6 cells (5×10^5) at two sites on the back. The tumor mass was obtained 2 h after the last injection of DT-5461. Cell proliferation was assayed in the presence or absence of the control IgG or anti-mTNF-α mAb in terms of [^{3}H]-thymidine uptake. rmTNF-α was used as a positive control. The values in parentheses represent % inhibition compared with control culture.
a) $P<0.01$ as compared with the untreated control.

Inhibitory effect of endogenous TNF-α induced by DT-5461 on endothelium proliferation Endothelial cell proliferation is thought to be essential for the capillary formation from host vascular beds in the process of neovascularization.[4] TNF-α inhibits the proliferation of vascular endothelium as well as tumor cells,[24,25] and consequently suppresses tumor-associated neovascularization.[8] We next investigated the effects on the proliferation of RLE cells *in vitro* by endogenous TNF-α in the serum and tumor homogenate from DT-5461-treated mice. As shown in Table III, sera produced after DT-5461 administration showed little or no inhibitory effect upon the proliferation of RLE cells. On the other hand, the inhibitory effect upon endothelium proliferation of the tumor homogenate from DT-5461-treated mice was remarkable, being similar to that of rmTNF-α at a concentration of 100 ng/ml, compared with that of the untreated control (Table IV). Moreover, this inhibitory effect of tumor homogenate was completely neutralized by anti-mTNF-α mAb (Table V). These results indicated that local production of endogenous TNF-α induced by DT-5461 in the tumor lesion contributes to the inhibition of endothelium proliferation.

Influence of anti-mTNF-α antiserum on inhibitory effect of tumor-associated angiogenesis by DT-5461 To determine the relationship between the anti-angiogenic effect of DT-5461 and the production of endogenous TNF-α,

Table VI. Influence of Anti-mTNF-α Antiserum on the Inhibitory Effect of Tumor-induced Angiogenesis by DT-5461

Administered with:	Angiogenesis (mean no. of vessels±SD)	Tumor size (mm)
Untreated (PBS)	22±1	5.2±0.3
+normal rabbit serum	21±4	5.2±0.5
+rabbit anti-mTNF-α antiserum	21±4	5.0±0.8
DT-5461	11±2[b]	3.8±0.3[b]
+normal rabbit serum	14±2[b]	3.8±0.4[b]
+rabbit anti-mTNF-α antiserum	24±4	4.2±0.6[a]
rmTNF-α	10±1[b]	2.8±1.1[b]
+normal rabbit serum	12±3[b]	3.5±0.3[b]
+rabbit anti-mTNF-α antiserum	20±2	5.3±0.9

Three C57BL/6 mice per group were given an i.v. injection of 50 μg/head of DT-5461 or an i.t. injection of 50 ng/head rmTNF-α simultaneously, with or without an i.t. injection of normal rabbit serum or rabbit anti-mTNF-α antiserum the day after an i.d. inoculation of B16-BL6 cells (5×10^5) at two sites on the back. Three days later, the mice were killed and the skin was separated from the underlying tissues. Angiogenesis was quantified by counting the number of vessels oriented toward the tumor mass and tumor size was measured. rmTNF-α was used as a positive control.
a) $P<0.01$, *b*) $P<0.001$ as compared with the untreated control.

Table VII. Inhibition of Spontaneous Lung Metastasis of B16-BL6 Melanoma Cells by DT-5461

Administered i.v. with:	Primary tumor size on day 21 (mean±SD)	No. of lung metastases on day 35 mean±SD (range)
Untreated (PBS)	11.1±0.7	44±12 (31–56)
DT-5461	8.9±0.6[a)]	14±8 (5–26)[a)]

Eight C57BL/6 mice per group were inoculated s.c. with 5×10^5 B16-BL6 melanoma cells into the right hind footpad and administered i.v. with DT-5461 on days 1, 5, 9, 13, 17, 22, 26, 30 and 34 after tumor inoculation. Primary tumors were surgically removed on day 21 and the mice were killed 35 days after tumor inoculation.
a) $P<0.01$ as compared with the untreated control.

we examined whether anti-mTNF-α antiserum neutralized the inhibitory effect of DT-5461 on tumor-associated angiogenesis (Table VI). Rabbit anti-mTNF-α antiserum as well as normal serum did not influence either the formation of capillary vessels, or the increase of tumor growth. On the other hand, rabbit anti-mTNF-α antiserum completely abrogated the inhibitory effect of DT-5461 upon angiogenic response, whereas the inhibitory effect on the tumor mass growth by DT-5461 was only partly diminished. These results indicated that suppression of tumor-associated angiogenesis by DT-5461 is partly due to the production of endogenous TNF-α.

Inhibition of spontaneous lung metastasis of B16-BL6 melanoma by DT-5461 The above study demonstrated that multiple DT-5461 administration suppressed tumor neovascularization. We examined the inhibitory effect of DT-5461 on the primary tumor growth and lung metastasis of B16-BL6 cells in a spontaneous metastasis model in mice. Table VII shows that multiple injections of DT-5461 significantly inhibited the primary tumor growth and lung metastasis of B16-BL6 cells compared with the untreated control. These results suggested that the suppression of tumor neovascularization and tumor growth by DT-5461 contributes to the inhibition of tumor metastasis.

DISCUSSION

The development of a vascular network appears to be involved in the progressive growth of solid tumors at primary or secondary sites and in the process of metastasis from the original site to specific organs. The suppression of tumor-associated angiogenesis might therefore provide a means of inhibiting tumor growth and metastasis. In this study, we examined the effect of DT-5461, a synthetic lipid A analogue with low endotoxicity, on the neovascularization induced by solid tumors in syngeneic mice. We showed that the i.v. administration of LPS the day after i.d. inoculation of B16-BL6 cells markedly reduced the number of capillary vessels oriented toward the tumor mass and the tumor growth 3 days after tumor inoculation as compared with untreated controls (Table I). The single administration of DT-5461 also significantly inhibited angiogenic response and tumor mass growth (Table I). These results indicated that the administration of DT-5461 as well as LPS during the early phase of neovascularization is effective for preventing the capillary formation. Furthermore, two or three injections of DT-5461 at intervals of 4 days caused a marked inhibition of tumor-induced angiogenesis and tumor growth (Table II). This result is consistent with our previous report that multiple injections of DT-5461 inhibited tumor metastasis.[20] These phenomena implied that the suppression of tumor-induced neovascularization is associated with the inhibition of tumor metastasis.

We showed that multiple injections of DT-5461 induced low levels of serum TNF-α in comparison with LPS in tumor-bearing mice (Table III). We previously reported that DT-5461 possesses less inducing activity for TNF-α than LPS or lipid A in murine peritoneal macrophages[26,27] or human peripheral blood monocytes (Sato *et al.*, manuscript submitted for publication). Furthermore, we observed that a single administration of DT-5461 resulted in no detectable production of serum endogenous TNF-α in mice (data not shown). These results indicated that the reason why systemic injections of DT-5461 induced little or no production of serum endogenous TNF-α may be low TNF-α-inducing activity in monocytes/macrophages. The inflammatory cytokines derived from monocytes/macrophages, including TNF-α, have been shown to be principal mediators of sepsis and endotoxic shock.[15] Thus, the lack of detectable toxic actions of DT-5461 on the host may be partly due to the low induction of TNF-α secretion. It is generally accepted that endogenous TNF-α mediates LPS-induced hemorrhagic necrosis and the regression of established solid tumors in mice.[16] The i.v. injection of LPS into mice induced TNF-α in the tumor lesion, and endogenous TNF-α is an essential participant in LPS-induced regression of an established SA1 sarcoma.[17] We observed that multiple injections of DT-5461 as well as LPS induced the high production of endogenous TNF-α in tumors (Table III). Kumazawa *et al.* also reported that the local induction of endogenous TNF-α may be responsible for the antitumor effects of DT-5461 (personal communication). It is still unclear from where the intratumor TNF-α originates, but it seems almost certain that it is not derived from blood. This interpretation is supported by the additional findings that more TNF-α was detectable in the tumor than in serum. North and Havell[17] suggested that tumor-infiltrating macrophages

(TIM) are responsible for LPS-induced production of intratumor TNF-α. We observed that DT-5461 did not directly stimulate B16-BL6 cells to secrete TNF-α *in vitro* (data not shown). These results imply that DT-5461 activates TIM to secrete TNF-α in tumor tissue. The reason why the systemic administration of DT-5461 induced marked amounts of endogenous TNF-α in tumors but only small amounts in serum of tumor-bearing mice is still unclear, but one possible reason is that DT-5461 may more effectively potentiate TIM than peripheral blood monocytes to release TNF-α. Further study is needed to clarify the mechanism of intratumor TNF-α production in detail.

Endothelial cell proliferation, which occurs within a capillary sprout, is a crucial event in the process of neovascularization.[4] The mechanism of the inhibitory effect of DT-5461 on capillary formation may be associated with the host-mediated response, since DT-5461 was not directly cytotoxic against endothelial cells, nor did it affect the cell growth (data not shown). It is well known that TNF-α possesses an antiproliferative effect upon endothelial cells *in vitro*.[24, 25] We observed that sera produced after DT-5461 administration did not inhibit endothelial proliferation (Table IV). On the other hand, the tumor homogenate from DT-5461-treated mice, as well as rmTNF-α markedly inhibited endothelial proliferation and these effects were completely abrogated by anti-mTNF-α mAb (Tables IV and V). These results suggested that inhibition of endothelial proliferation by DT-5461, mediated through the production of endogenous TNF-α in tumor sites, contributes in part to the inhibition of new capillary formation.

LPS induces hemorrhagic necrosis and regression of established tumors in some experimental animal models and LPS-induced suppression of tumors may be caused by damage to the tumor vasculature, rather than by the direct destruction of tumor cells.[16, 17] It is generally accepted that the tumor-induced vasculature is structurally immature and vulnerable as compared with normal vascular vessels,[28] and tumor blood vessels may be more susceptible to TNF-α than normal blood vessels. On the other hand, the role of TNF-α in the pathophysiology of angiogenesis is still controversial. Fajardo *et al.*[29] reported that low doses of TNF-α induced angiogenesis, whereas high doses inhibited it. These results implied that the paradoxical effects of TNF-α on angiogenesis depend on its local tissue concentration. We demonstrated that the anti-angiogenic effect of DT-5461 was completely abrogated by the i.t. injection of rabbit anti-mTNF-α antiserum (Table VI). This suggested that high TNF-α production induced by DT-5461 in tumor lesions contributes to the inhibition of tumor-induced angiogenesis. We also observed that the i.t. injection of rabbit anti-mTNF-α antiserum failed to neutralize completely the inhibitory effect of DT-5461 on tumor mass growth (Table VI). This result indicates that a TNF-α-independent mechanism is involved in the DT-5461-induced regression of tumor growth. In fact, we observed that the administration of DT-5461 to mice augmented the tumoricidal activity of murine peritoneal macrophages and the NK activity of murine splenocytes against B16-BL6 murine melanoma or RL male 1 murine leukemia cells, respectively.[26] Furthermore, Akimoto *et al.* suggested that IFN α/β and γ are involved in the antitumor mechanism of DT-5461.[27] The inhibitory effect of DT-5461 on tumor growth may thus be associated with the participation of a host immune defense mechanism that is not TNF-α-dependent.

It has been shown that TNF-α possesses an antimetastatic effect in certain murine metastasis models.[30] The multiple i.v. administration of DT-5461 significantly reduced the number of lung tumor colonies, as well as the primary tumor size in a spontaneous metastasis model (Table VII). These results suggested that the inhibition of tumor growth and metastasis by DT-5461 is due to the suppression of tumor-induced neovascularization in both primary and secondary tumors. The mechanism responsible for the inhibition of tumor growth and metastasis caused by the suppression of vasculogenesis may be functional collapse of the tumor vasculature, including loss of blood supply or inhibition of shedding metastatic cells, from primary or secondary sites. On the other hand, recent evidence has shown that a single injection of TNF-α before i.v. inoculation of tumor cells caused a significant augmentation of tumor metastasis in experimental metastasis models.[31] One possible explanation for these paradoxical observations on the effect of TNF-α on tumor metastasis may be differences in the microenvironment surrounding the tumor and host cells, though the experimental designs also differ.

In conclusion, we have demonstrated that the inhibitory effect of endogenous TNF-α induced by DT-5461 upon endothelium proliferation may contribute in part to the suppression of tumor-induced angiogenesis, and consequently lead to the inhibition of tumor growth and metastasis. The mechanism of the regression of tumor growth and metastasis by DT-5461 remains to be determined in detail.

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