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Advances in Brief

Responses to Antiangiogenesis Treatment of Spontaneous Autochthonous Tumors and Their Isografts[1]

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Abstract

Preclinical studies typically use human tumor xenografts or murine tumor isografts. Tumor growth may be accelerated by *in vivo* passage, thus making these tumors more sensitive to some therapies than the original tumors. In the present study, by comparing the effects of DC101, an antimurine vascular endothelial growth factor receptor 2 (VEGFR2) monoclonal antibody, on spontaneous autochthonous tumors and their early generation transplants, we show that this growth acceleration is diminished by DC101 treatment. Spontaneous autochthonous tumors in aged C3H mice consisted of s.c. sarcomas and adenocarcinomas, and their growth rate was accelerated by *in vivo* passages. Anti-VEGFR2 treatment decreased vessel density, increased apoptosis, and reduced tumor growth in large (500 mm^3) spontaneous autochthonous tumors. Anti-VEGFR2 treatment significantly delayed tumor growth and extended animal survival. Tumor growth acceleration by *in vivo* passage was diminished by DC101 treatment. To our knowledge, this is the first evaluation of antiangiogenic therapy in a spontaneous autochthonous tumor model, which may more closely resemble human tumors. Additionally, this is the first study to compare treatment response between the parental tumor and its isografts. Although passaged tumors behave differently, it is encouraging that the tumor growth rates under DC101 treatment are comparable among different passage generations.

Introduction

Tumor angiogenesis is a result of dynamic interactions between the tumor cells and the host (1, 2). Indeed, models using tumors implanted in orthotopic (natural) organ sites reveal the importance of host-tumor interactions in tumor angiogenesis and response to therapies (2–6). However, experimental tumors implanted in healthy young animals may behave differently compared with tumors that arise spontaneously, mostly in aged individuals. Typically, experimental tumors are derived from spontaneous autochthonous tumors of various origins and propagated in animals (in most cases mice) and/or established as cell lines *in vitro*. However, *in vivo* passage of tumors may accelerate their growth rate, regardless of whether they are derived from spontaneous tumors, isografts, or an established cell line. In this study, we evaluated the efficacy of antiangiogenesis treatment with DC101, an anti-VEGFR2[3] monoclonal antibody (7) on spontaneous autochthonous tumors (sarcomas and adenocarcinomas) that arose in aged female C3Hf/Sed mice (8–10). We also compared the responses to DC101 of tumor isografts and tumors from cell lines derived from the spontaneous tumors. Accelerated growth rates of tumors passaged *in vivo* were diminished by DC101 treatment.

Materials and Methods

Treatment of Spontaneous Autochthonous Tumors. Inbred female C3Hf/Sed mice (murine mammary tumor virus free), retired breeders, were the source of tumors for this study. The mice were maintained in our defined flora, pathogen-free animal facility and allowed to live their normal life span. These mice were screened weekly for tumor development by visual inspection and palpation. Once a tumor was found, tumor size was measured every 3 days along three diameters with a vernier caliper. Tumor volume was calculated as $4/3\pi r^3$, where r is half the average of the three diameters. When the tumor volume reached approximately 500 mm^3, the animal was randomly assigned to either anti-VEGFR2 antibody (DC101) or control antibody treatment. The treatment group received DC101 (ImClone Systems Inc., New York, NY), 40 mg/kg, i.p., every 3 days. The control group received nonspecific rat IgG antibody (ImClone Systems Inc.), 40 mg/kg, i.p., every 3 days. In the initial study, DC101 and control antibody administration was continued for 24 days. For long-term tumor response studies, the treatment was continued until the tumor exceeded 4000 mm^3 or until the animal became moribund, at which point the animal was sacrificed, and macroscopic autopsy was performed. If multiple tumors were found simultaneously, the largest tumor was retained, and the others were resected. If additional tumors arose after the detection of the first tumor, they were likewise resected.

Histological Analysis of Spontaneous Autochthonous Tumors. In the initial study, animals were sacrificed 24 days after initiation of the treatment for histological examination of tumor vessel density and tumor cell apoptosis. Tumors were fixed by vascular perfusion of mice using 4% paraformaldehyde in PBS (11). The tumors were then removed and split. Half of the tumor was fixed in 10% neutral buffered formalin overnight at 4°C, followed by embedding in paraffin. The other half was postfixed in 4% paraformaldehyde for 1 h at 4°C, washed in PBS, cryoprotected in 30% sucrose overnight, and finally frozen in OCT embedding compound.

H&E staining was done using paraffin sections to determine tumor histology. The vessels in the sarcomas were stained from OCT sections using FITC-conjugated rat antimouse CD31 monoclonal antibody (clone MEC13.3; BD PharMingen, San Diego, CA) and developed with a DAB kit (Zymed, San Francisco, CA) per the manufacturer's instructions. The vessels in the adenocarcinomas were stained for the mouse pan-endothelial antigen MECA from paraffin sections using rat antimouse MECA-32 (1:200; PharMingen and developed with DAB (DAKO DAB solution).

TUNEL staining was performed from paraffin sections using an *In Situ* Cell Death Detection Kit (Roche Molecular Biochemicals, Indianapolis, IN). Images of bright-field and fluorescence-stained sections were viewed on Axioskop (Zeiss, Oberkochen, Germany) and digitized using a Sony camera and Scion CG-7 framegrabber. For vessel density quantification, 10 random fields per sample at ×200 magnification were analyzed for number of DAB-positive vessels using Corel PhotoPaint Version 2.0 (Ontario, Canada). For TUNEL index, five ×400 magnification fields per sample of high-density, TUNEL-positive areas not located in necrotic regions were analyzed. In addition, the same field that was stained with Hoechst (Molecular Probes, Eugene, OR) was also captured for analysis. Using Corel PhotoPaint Version 2.0, the number of TUNEL-positive pixels (green pixels) per field was calculated and averaged

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[3] The abbreviations used are: VEGFR2, vascular endothelial growth factor receptor 2; DAB, diaminobenzidine tetrachloride; RI, response index; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

over 5 fields/specimen. The number of Hoechst-positive pixels (blue pixels) per field was also calculated and averaged over 5 fields/specimen. The TUNEL index equals the number of green pixels divided by the number of blue pixels times a factor of 100.

Propagation and Treatment of Isografts and Cell Line-derived Tumors. When a spontaneous autochthonous tumor (no treatment) grew to approximately 15 mm in diameter, the tumor was resected. Tumor fragments of approximately 2 mm were sliced from the nonnecrotic parts of the tumor and then immediately transplanted into 15–20 recipient mice. Female C3Hf/Sed mice, approximately 6 months old, were used as recipients. The tumor fragment was transplanted in the site corresponding to the parental spontaneous autochthonous tumor. They were designated as the first passage isograft (F_1). Tumor size measurement was initiated as in the spontaneous autochthonous tumors, when the tumors became detectable. The first tumor to reach 15 mm was transplanted to the next set of 15–20 mice as F_2. Transplantation was only attempted once. Our intention was to select for the fastest growing tumors with better transplant take rates. The rest of the animals were assigned to either DC101 or control antibody treatment when the tumor exceeded 500 mm^3. Treatment and size measurements were done as in the spontaneous autochthonous tumors. Also, a cell line was established from the F_5 sarcoma. The tumors derived from this cell line were also serially passaged and treated with a protocol similar to that used for the isografts. We compared RI, defined as (DC101 survival)/(control survival), between different passages of isografts and cell line-derived tumors.

Plasma Sample Collection. Plasma samples were collected at the time of euthanasia. Approximately 1.0 ml of blood was withdrawn by cardiac puncture and collected in Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) containing 0.1 ml of EDTA. The blood was immediately centrifuged at 426 $\times$ *g* for 10 min at 4°C, followed by additional centrifugation at 4165 $\times$ *g* at 4°C. Plasma samples were stored in aliquots at –70°C.

DC101 Neutralization Assay. ELISA plates were coated with 200 ng of DC101 in PBS for 2 h at room temperature. Plates were blocked with 5% BSA in PBS. Plasma samples were serially diluted on the plate and incubated for 1 h at room temperature. After an extensive wash with PBS/0.1% Tween 20, 200 ng of Flk-1 alkaline phosphatase were added to the plates and incubated for 1 h at room temperature. After more washes, the plates were developed using *p*-nitophenyl phosphate tablets (Sigma N-2765) in substrate buffer (1 M diethanolamine, 0.5 mM MgCl, 10 mM a-homoarginine, and 0.5 mg/ml BSA). The assay was read at a wavelength of 405 nm on an Emax precision microplate reader (Molecular Devices).

Anti-DC101 ELISA. ELISA plates were coated with 200 ng of DC101 in PBS for 2 h at room temperature. Plates were blocked with 5% milk and 5% BSA in PBS. Plasma samples were serially diluted on the plate and incubated for 1 h at room temperature. After an extensive wash with PBS/0.1% Tween 20, a rat anti-mouse Igκ horseradish peroxidase (BioSource International) secondary antibody was added to the plates and incubated for 1 h at room temperature. After more washes, the plates were developed using a tetramethyl-benzidine peroxidase system (Kirkegaard and Perry). Assay was read at a wavelength of 450 nm on an Emax precision microplate reader (Molecular Devices).

Statistical Analysis. Data are shown as mean $\pm$ SD. Data between groups were compared by Mann-Whitney *t* test unless otherwise noted (StatView; Abacus, Berkeley, CA). Exponential curve fittings and linear regression analysis were done using StatView. *P*s $<$ 5% were considered significant.

Results and Discussion

Spontaneous Autochthonous Tumors Consisted of s.c. Sarcomas and Adenocarcinomas. Thirty animals in total, between 21 and 25 months old, entered the study. The tumors all arose in the subcutaneum. Fourteen animals were entered into the initial study, and the animals were euthanized at 24 days after the start of the treatment for histological analyses of vessel density and apoptosis. The tumors were diagnosed as sarcomas (9 tumors: DC101, $n = 5$; control, $n = 4$) and adenocarcinomas (5 tumors: DC101, $n = 3$; control, $n = 2$). Then, a separate group of 16 animals was entered into long-term treatment response study. After the completion of the study, the tumors were histologically diagnosed as 15 sarcomas (DC101, $n = 7$; control, $n = 8$) and 1 adenocarcinoma (DC101, $n = 1$). There were no apparent differences in the growth curves between the two groups from the time of detection until the initiation of treatment (Fig. 1*a*). The slope calculated from exponential curve fittings showed no significant difference [0.124 $\pm$ 0.054 (DC101 group) *versus* 0.144 $\pm$ 0.065 (control group); $P = 0.59$, Mann-Whitney *t* test].

Anti-VEGFR2 Treatment Decreased Vessel Density, Increased Apoptosis, and Reduced Tumor Growth in the Spontaneous Autochthonous Tumors. Within randomly assigned 14 spontaneous autochthonous tumors, all tumors treated with DC101 were smaller than the tumors in the control group regardless of histological types at 24 days after the initiation of treatment (Fig. 1*b*; Table 1). Representative histological images of each tumor type are shown in Fig. 2. H&E staining showed that the sarcomas were mostly fibrosarcomas with the spindle cells arranged in interlacing bundles (Fig. 2*a*). The adenocarcinomas had typical glandular structures and were judged to be of mammary origin (Fig. 2*b*). Histological vessel density measurement with anti-CD31 or MECA-32 immunostaining indicated that anti-VEGFR2 treatment with DC101 significantly inhibited angiogenesis in spontaneous autochthonous tumors regardless of their histological types (Fig. 2, *c*–*f*; Table 1). Vessel densities in DC101-treated sarcomas and adenocarcinomas were 3- and 3.5-fold lower than those of the corresponding control tumors, respectively. Furthermore, TUNEL staining revealed that apoptosis rates were significantly higher in DC101-treated tumors compared with the control tumors in both histological types (Fig. 2, *g*–*j*; Table 1). There were 3- and 50-fold increases in TUNEL index in the DC101-treated sarcomas and adenocarcinomas, respectively. As a result, DC101-treated tumors were significantly smaller than the control tumors at 24 days after initiation of treatment for both histological types (Table 1).

Anti-VEGFR2 Treatment Significantly Delayed Tumor Growth and Extended Animal Survival. In the long-term treatment response study, tumor growth was slower in the DC101 group compared with the control group, with the exception of one sarcoma (Fig. 1*c*). Because there was only one entry of adenocarcinoma for long-term study, we excluded adenocarcinoma from subsequent analysis. In the sarcomas, there were no apparent differences in the initial growth curves between the two treatment groups. This is based on analysis of the growth curves from the time of detection until the initiation of treatment as indicated by the slope calculated from exponential curve fittings [0.154 $\pm$ 0.028 (DC101 group) *versus* 0.177 $\pm$ 0.068 (control group); $P = 0.95$, Mann-Whitney *t* test]. The animal survival (time period from initiation of treatment until exclusion of the animal) was significantly extended in the DC101 group compared with the control group (43 $\pm$ 12 days in the DC101 group *versus* 28 $\pm$ 12 days in the control group; $P = 0.03$, Mann-Whitney *t* test). No apparent adverse effects directly attributable to treatment were observed during the study.

These effects of DC101 in established spontaneous autochthonous tumors are in general agreement with the previous studies with a variety of implanted human and murine tumors (12–15). DC101 treatment did not exhibit appreciable effects on the incidence of metastasis and/or another primary tumor formation in our spontaneous autochthonous tumor model. Macroscopic metastases were seen at autopsy in the lungs in the fastest-growing tumors (two sarcomas, one in each treatment group; Fig. 1*c*, *#5* and *#6*). In four animals a second s.c. tumor was detected during the study and removed immediately upon detection. These were one adenocarcinoma in the control group (initial study; Fig. 1*b*, *#1*) and three sarcomas in the DC101-treated group (long-term study; Fig. 1*c*, *#2*, *#3*, and *#4*). These secondary tumors were approximately 4–5 mm in diameter when they were found (9–18 days after initiation of the treatment). Considering the relatively slow growth kinetics of these spontaneous tumors and the detection limit of tumors in this study, it is likely that these tumors were already macroscopically present at the start of treatment. Hence, it is difficult to interpret the potential effect of DC101 on secondary

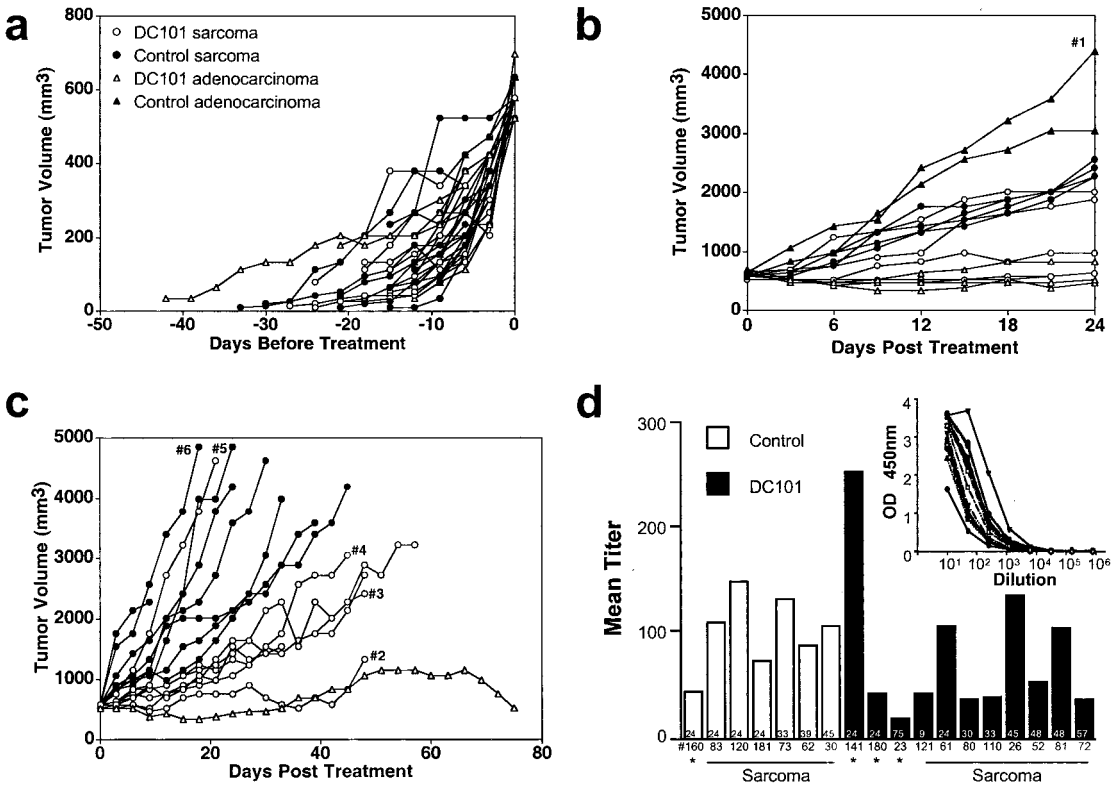

Fig. 1. *a*, the growth curves of individual tumors from the time of detection until the initiation of treatment (day 0). ○, DC101-treated sarcoma, $n = 12$; ●, control sarcoma, $n = 12$; △, DC101-treated adenocarcinoma, $n = 4$; ▲, control adenocarcinoma, $n = 2$. *b*, tumor volume after initiation of treatment up to 24 days. ○, DC101-treated sarcoma, $n = 5$; ●, control sarcoma, $n = 4$; △, DC101-treated adenocarcinoma, $n = 3$; ▲, control adenocarcinoma, $n = 2$. A second tumor (adenocarcinoma) was detected in one control adenocarcinoma (*#1*) 3 days before initiation of treatment and immediately resected. *c*, tumor volume after initiation of treatment (day 0). ○, DC101-treated sarcoma, $n = 7$; ●, control sarcoma, $n = 8$; △, DC101-treated adenocarcinoma, $n = 1$. A second tumor was detected in the DC101-treated sarcomas (*#2, #3,* and *#4*). The second tumors were detected and immediately resected at 18, 12, and 9 days after initiation of treatment in *#2, #3,* and *#4*, respectively. The second tumors were all sarcomas. Macroscopic metastases were seen at autopsy in the lung in two sarcomas, one in the DC101 group (*#5*) and one in control group (*#6*). *d*, mean antirat (DC101) immunoglobulin titers in plasma samples taken from treated mice. No significant differences in antirat immunoglobulin titers were observed in the plasma samples by two-way ANOVA analysis between DC101-treated animals (■) and control antibody-treated mice (□) or sarcoma and adenocarcinoma. The columns show mean titer of each plasma sample (sample numbers are designated *below* each column). *Numbers inside the column* indicate duration of treatment (in days). There is no correlation between antibody titers and treatment days ($P = 0.3192$ by linear regression analysis). ∗, samples from adenocarcinoma-bearing mice. *Inset* shows titration of antirat immunoglobulin responses in plasma of all treated mice.

tumors. It is noteworthy that no notable impairment of wound healing was observed in the resection sites of second tumor in the animals that received DC101 treatment. This is consistent with previous documentation that antiangiogenesis treatment interferes with wound vessel formation (16, 17) but that it does not necessarily affect the overall macroscopic wound healing process (16). Finally, despite significant tumor growth delay with DC101 treatment, eventually all tumors reached maximum tumor size and/or the animals became moribund and were sacrificed in the long-term study. The production of anti-DC101 antibody in immunocompetent mice may have interfered with the effect of DC101 during long-term treatment. In the present study, a low level of antirat IgG antibodies was detected in the plasma of the animals treated with control rat IgG or DC101 (Fig. 1*d*). However, no neutralizing anti-DC101 immunoglobulin was detected in any of plasma samples tested. Nevertheless, we cannot rule out the possibility that an antirat immune response may have diminished the activity of DC101 over the long treatment period used in our studies. It should be noted that DC101 delayed transplanted tumor growth in immunocompetent mice (C57BL/6 and BALB/c) in previous studies despite the presence of antirat immunoglobulin responses (13). Therefore, we believe that our studies demonstrate activity of DC101 in C3H mice despite evidence of some antibody production. It has been also documented that the ability to produce specific antibodies after antigenic challenges is decreased in old mice (18). This may partly explain the lower plasma titers and the lack of apparent DC101 neutralization found in our study.

Relatively large tumor size (very advanced spontaneous autochthonous tumors) at the start of treatment in the present study (500 mm^3) needs to be accounted for. This size was chosen to allow sufficient observation time for the growth of individual tumors from the time of detection until the initiation of treatment. DC101 treatment delayed tumor growth significantly even in this unfavorable experimental design.

Table 1 *Comparisons of vessel density, TUNEL index, and tumor size at 24 days after initiation of treatment*

Vessel density and tumor size were significantly decreased, and TUNEL index was significantly increased in the DC101-treated sarcomas compared with the controls after 24 days of treatment.

	Sarcoma		Adenocarcinoma	
	Control ($n = 4$)	DC101 ($n = 5$)	Control ($n = 2$)	DC101 ($n = 3$)
Vessel density (vessels/field)	43.1 ± 15.1	16.1 ± 8.4[a]	94.6 ± 64.2	27.0 ± 7.5
TUNEL index (%)	0.138 ± 0.12	0.466 ± 0.213[a]	0.031 ± 0.029	1.559 ± 2.025
Tumor size (mm^3)	2389 ± 139	1230 ± 673[a]	3726 ± 953	609 ± 194

[a] $P < 0.05$ *versus* sarcoma control. The same tendency was observed in the adenocarcinomas, but the lack of animal number precluded statistical analysis.

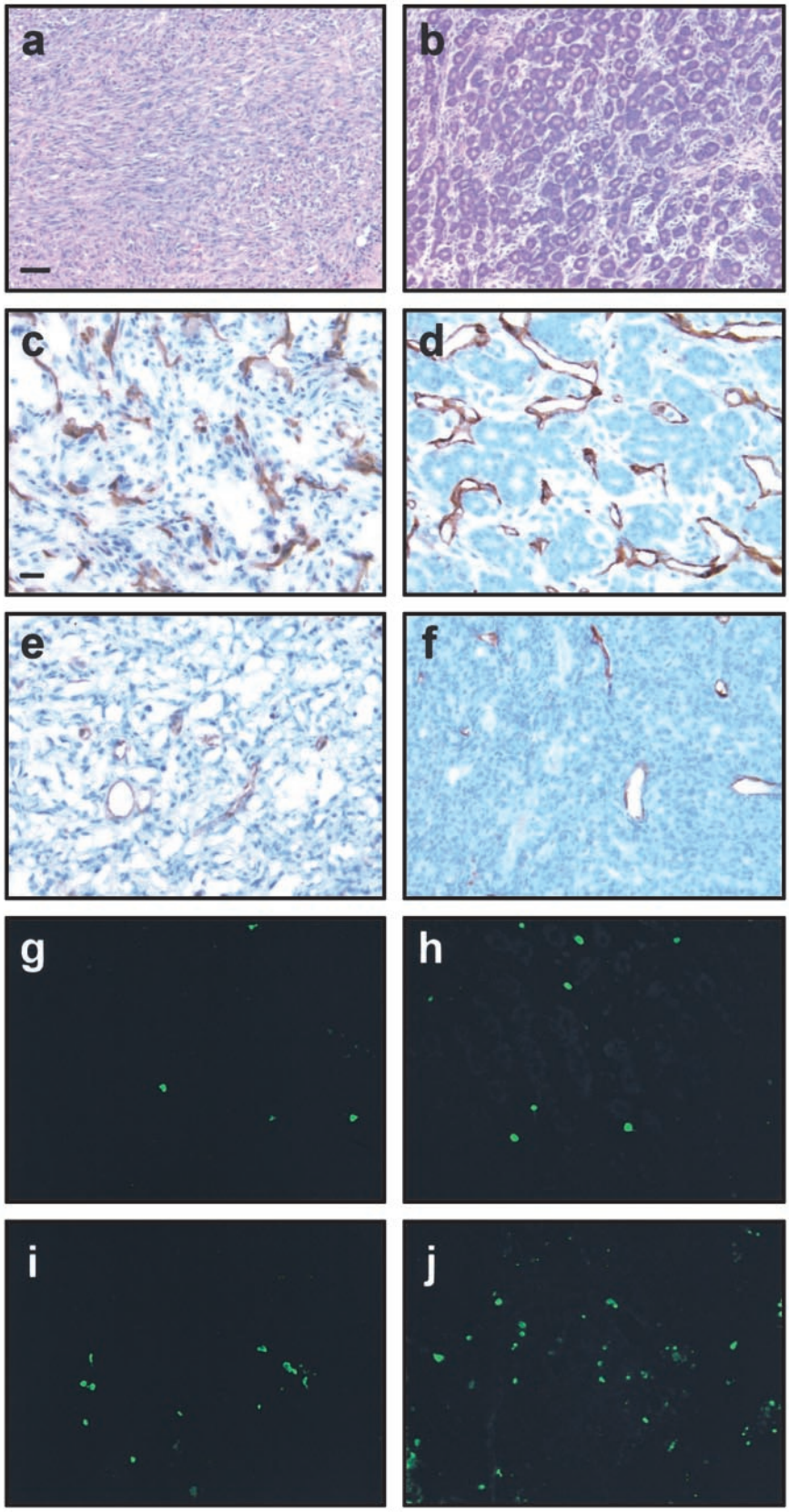

Fig. 2. Representative histological images of sarcomas (*a*, *c*, *e*, *g*, and *i*) and adenocarcinomas (*b*, *d*, *f*, *h*, and *j*) in the spontaneous tumors. H&E staining (*a* and *b*), CD31 staining (*c* and *e*), MECA-32 staining (*d* and *f*), and TUNEL staining (*g*−*j*) 24 days after the start of control antibody treatment (*c*, *d*, *g*, and *h*) or DC101 treatment (*e*, *f*, *i*, and *j*). The *scale bar* in *a* indicates 50 μm in *a* and *b*; the *scale bar* in *c* indicates 20 μm in *c*−*j*.

Tumor Growth Acceleration by *in Vivo* Passage Was Diminished by DC101 Treatment. Ten tumors were transplanted (five sarcomas and five adenocarcinomas). Of these 10 tumors, tumor transplant take was achieved for 2 tumors (*i.e.*, one sarcoma and one adenocarcinoma). These two were serially transplanted *in vivo*, selecting for faster-growing isografts. In the sarcoma, we established a cell line from the fifth passage isograft and then passaged the tumor derived from that cell line. As reported previously (19), these *in vivo*

Table 2 *Comparison of survival and RI between the different in vivo passages of isografts and cell line tumors*

The increase in growth rate by *in vivo* passage was diminished by DC101 treatment. Survival is defined as the time from initiation of treatment until the exclusion of the animal by tumor size or physical condition. RI is calculated as (DC101 survival)/(control survival).

A. Sarcoma

	Survival (days)		
	DC101	Control	RI
Isograft			
F_1	23 ± 5	17 ± 2	1.4 ± 0.3
F_3	33 ± 2[a]	15 ± 3	2.2 ± 0.5[a]
F_5	31 ± 3[a]	12 ± 4[a]	2.6 ± 0.9[a]
F_8	30 ± 2[a]	11 ± 2[a,b]	2.7 ± 0.5[a]
F_{11}	31 ± 3[a]	10 ± 2[a,b,c]	3.1 ± 0.7[a,b,c]
Cell line tumor			
F_5 cell-P_1	33 ± 3	17 ± 2	1.9 ± 0.3
F_5 cell-P_3	30 ± 1	15 ± 2	2.0 ± 0.3
F_5 cell-P_5	34 ± 2[b]	13 ± 2[a]	2.6 ± 0.4[a,b]
Spontaneous sarcomas	43 ± 12	28 ± 12	1.5 ± 0.8

B. Adenocarcinoma

	Survival (days)		
	DC101	Control	RI
Isograft			
F_1	40 ± 4	22 ± 1	1.8 ± 0.2
F_3	30 ± 2[d]	15 ± 2[d]	2.0 ± 0.3
F_5	28 ± 1[d]	12 ± 1[d,e]	2.3 ± 0.2[d,e]
F_8	30 ± 1[d]	9 ± 1[d,e,f]	3.3 ± 0.4[d,e,f]

[a] $P < 0.05$ *versus* F_1 or P_1 in the corresponding group.
[b] $P < 0.05$ *versus* F_3 or P_3 in the corresponding group.
[c] $P < 0.05$, F_5 *versus* F_{11} in the corresponding group.
[d] $P < 0.05$ *versus* F_1 in the corresponding group.
[e] $P < 0.05$ *versus* F_3 in the corresponding group.
[f] $P < 0.05$, F_5 *versus* F_8 in the corresponding group.

passages actually yielded faster-growing tumors (both sarcomas and adenocarcinomas; Table 2). Interestingly, the efficacy of DC101 was not significant for F_1 with a survival time of 23 days as compared with 17 days for the control animals. In comparison for F_2–F_5, the survival times in the DC101-treated mice were in the 30–34-day range. However, for the control mice, survival time decreased steeply with passage number (*i.e.*, $17 \rightarrow 15 \rightarrow 12 \rightarrow 11 \rightarrow 10$). There was a similar finding for the passages of the cell line derived from *in vitro* cell line. Importantly, the same phenomenon was observed for the adenocarcinomas (see Table 2).

These data suggest that acceleration of tumor growth by *in vivo* passage may be due, at least in part, to selection of the most rapidly dividing tumor clonogen or mediated by VEGFR2 signaling. If the latter were the dominant mechanism, it is not clear whether it is due to up-regulation of ligand and/or receptor expression. Segregation of tumor as fragments is required for *in vivo* tumor passage. Because transplanted tumor fragments at first do not have their own blood vessels to support the large number of implanted cells, it is likely that cells (tumor, endothelial, and stromal cells) expressing higher levels of VEGFR2 would have advantages in such nutrient-deprived conditions (20, 21) and hence be selected. Thus, this acquired growth advantage might be abrogated in part by DC101 treatment.

Because treatment efficacy is commonly evaluated relative to the controls, care is warranted in the extrapolating results from studies based on higher-passage tumors, as indicated by the increase in RI (Table 2). The response can be further skewed if different passage generations are compared. However, it is encouraging that the difference in RI stays within 3-fold even with 11 passages. The spontaneous autochthonous tumors arising in C3Hf/Sed mice may be a useful preclinical model, especially for novel treatment approaches such as anti-VEGFR2 treatment, despite the facts that murine tumor studies are still not directly applicable to human patients and that epithelial tumors are more common in human malignancies. Moreover, at present, large-scale systematic treatment-response studies are difficult using this model, due to its limited availability. Genetically engineered mouse models offer new approaches to understanding carcinogenesis, tumor progression, and treatment response (22). The spontaneous autochthonous tumors may be used for additional studies delineating the differences between spontaneous autochthonous tumors and transplanted tumors, to improve understanding of the advantages and limitations of the currently available experimental tumor systems, and to use these limitations in the planning of clinical trials.

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