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ARGININE DEIMINASE AND OTHER ANTIANGIOGENIC AGENTS INHIBIT UNFAVORABLE NEUROBLASTOMA GROWTH: POTENTIATION BY IRRADIATION

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In spite of aggressive therapy, children suffering from neuroblastoma have a poor prognosis. Therapeutic failure is most often observed in neuroblastomas with unfavorable features, including amplification/over-expression of the *N-myc* oncogene, rapid growth, effective angiogenesis and/or the tendency to metastasize. Here, we have used cultured human neuroblastoma cells with such features and we have examined whether antiangiogenic agents alone or in combination with tumor irradiation inhibit their angiogenesis and growth *in vivo*. We report that antiangiogenic agents (arginine deiminase, SU5416 and DC101) inhibit *in vivo* growth of neuroblastomas with unfavorable properties and that these effects are potentiated by simultaneous irradiation. Combination of either agent with irradiation leads to a reduction in the absolute number of tumor vessels and of perfused tumor vessels. Combination of arginine deiminase or DC101 with irradiation does not increase tumor hypoxia. Our data demonstrate for the first time that arginine deiminase suppresses the growth of unfavorable experimental neuroblastomas and that this effect is potentiated by irradiation. We suggest that antiangiogenesis alone or in combination with established therapeutic regimen may improve the outcome of unfavorable neuroblastomas in a clinical setting.

Key words: *neuroblastoma; arginine deiminase; angiogenesis; irradiation; tumor hypoxia*

Neuroblastoma is the most common solid malignancy in children. It is derived from immature precursor cells of the sympathetic ganglia and is classified into 4 stages of increasing tumor dissemination.[1] At the time of diagnosis, most patients are in the prognostically unfavorable stages 3 and 4, where metastasis has occurred and clinical outcome is poor, even under the most aggressive treatment strategies.[2,3] Patients with neuroblastoma contribute to approximately 15% of childhood cancer deaths.[4,5]

The *N-myc* oncogene may contribute significantly to mortality, since it is often amplified in neuroblastoma and amplification correlates with advanced tumor stage and poor prognosis.[6–9] However, the molecular consequences of *N-myc* amplification have remained obscure. To shed light on these questions, we have developed a neuroblastoma model that originates from the human neuroblastoma cell line SH-EP. We have transfected SH-EP cells with a control vector or a vector that contains the human *N-myc* cDNA. We obtained a cell line with normal *N-myc* expression (SH-EP007, controls) and a cell line with more than 100-fold enhanced *N-myc* expression (WAC2). Unlike the controls, WAC2 cells exhibit features characteristic of cells derived from unfavorable neuroblastomas, including the ability to rapidly proliferate *in vitro* and *in vivo* and to induce angiogenesis in vivo.[10]

Angiogenesis, the formation of new vessels from existing ones, is an indispensable requirement for the growth and progression of solid malignancies. Once a tumor has reached a diameter of several millimeters, its growing demand for nutrients and oxygen can only be satisfied if the tumor can attract new blood vessels and establish a functional vascular network. During angiogenesis, existing capillary endothelial cells migrate towards a tumor, proliferate and divide, eventually giving rise to new blood vessels.[11–14] Some tumors can induce angiogenesis by activating proangiogenic molecules, including basic fibroblast growth factor (bFGF) with its receptors on the corresponding tumor vessels or vascular endothelial growth factor (VEGF) with its corresponding receptors Flt-1 (VEGFR1) and Flk-1/KDR (VEGFR2) on the corresponding tumor vessels.[14–17] Tumors may also induce angiogenesis by blocking antiangiogenic molecules. For example, they may down-regulate soluble Flt-1 and thereby prevent it from binding and neutralizing VEGF.[18–20] Experimental and clinical evidence indicates that *N-myc* over-expression induces angiogenesis in neuroblastoma.[10,21] Using our model of neuroblastoma cells ± enhanced *N-myc* expression,[10,22] we were able to demonstrate the presence of angiogenesis stimulators including VEGF in these cells.[23] In the same model, we were able to demonstrate that *N-myc* can down-regulate 3 angiogenesis inhibitors, which we identified as activin-A, interleukin-6 and leukemia inhibitory factor, and as a consequence thereof permits tumor angiogenesis.[24–27]

We and others have identified several other inhibitors of angiogenesis, including 2-methoxyestradiol and arginine deiminase.[28,29] Although both molecules inhibit angiogenesis, they can also inhibit the proliferation of tumor cells directly and are therefore classified as nonspecific angiogenesis inhibitors. In contrast, several other molecules have been synthesized that block angiogenesis specifically. For example, SU5416 blocks tyrosine kinase activity of the VEGF receptor 2 (VEGFR2),[30] while the monoclonal rat anti-mouse antibody DC101 blocks VEGFR2 activity by

Abbreviations. BBCE, bovine brain-derived capillary endothelial (cell); bFGF, basic fibroblast growth factor; BrdU, 5-bromo-2′-deoxyuridine; Flk-1, fetal liver kinase 1 (the specific name of murine VEGFR2); Flt-1, fms-like tyrosine kinase 1; HUVE, human umbilical vein endothelial (cell); KDR, kinase insert domain-containing receptor (the name of human VEGFR2); PBS, phosphate-buffered saline; pO_2, oxygen partial pressure; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

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blocking its extracellular domain.[31] Conventional therapy, *i.e.*, surgery, chemo- and radio-therapy, has improved outcome of children with neuroblastoma considerably. However, neuroblastomas with unfavorable properties, the most important being *N-myc* amplification and its consequences, often do not respond persistently to these treatments and eventually kill their hosts.[6,21] We have therefore speculated that antiangiogenesis alone or in combination with conventional strategies could help to improve outcome in such cases. As a neuroblastoma cell line that exhibits properties of unfavorable neuroblastomas, we selected the well-characterized human neuroblastoma cell line WAC2[10] because the latter readily grew *in vivo* after approximately 5 days, while cells with genuine N-myc amplification including IMR32 and Kelly grew only after an average incubation time of 50 days. Using WAC2 cells, we attempted to study the effects of 1 unspecific (arginine deiminase) or 2 specific (SU5416 and DC101) angiogenesis inhibitors alone or in combination with irradiation on the growth of human neuroblastomas *in vitro* and *in vivo*.

MATERIAL AND METHODS

Materials

Hoechst 33342 was purchased from Sigma Chemical Co. (St. Louis, MO), goat anti-type IV collagen from Southern Biotechnology (Birmingham, AL) and TRITC-labeled donkey anti-goat IgG from Jackson Immuno Research (West Grove, PA). DC101 and SU5416 were kindly donated by ImClone Systems, Inc. (New York, NY) and SUGEN, Inc. (South San Francisco, CA), respectively. Mycoplasma arginini (ATCC 23838) was purchased from the American Type Culture Collection (Rockville, MD). Arginine deiminase was purified from Mycoplasma arginini as previously described.[29] Its potent inhibitory effect on the growth of various cells was routinely verified and was destroyed by pretreatment with heat. Analysis of the purified protein by SDS polyacrylamide gel electrophoresis revealed a single band of molecular weight of 45 kDa. Neuroblastoma (WAC2) and vascular endothelial cells derived from bovine brain (BBCE) or human umbilical vein (HUVE) were from sources mentioned earlier.[28,32–34] Nude mice (nu/nu of NMRI inbred background) were obtained from the central animal facility of University of Essen and were kept under pathogen-free conditions. The animals entered the experiment at an age of 7–9 weeks. All animal experiments were in accordance with animal protection laws and had previously been approved by the local animal ethics committee.

Cell culture

Neuroblastoma cells were maintained in culture dishes containing medium (RPMI 1640) supplemented with fetal calf serum (10%), antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin and 1.25 μg/ml amphotericin β) and G418 (200 μg/ml) to select continuously for cells with enhanced *N-myc* expression.[10] BBCE or HUVE cells were cultured in dishes containing medium (DMEM or Medium 199, respectively) supplemented with newborn calf serum (10%) or fetal calf serum (20%), respectively, and antibiotics.[28,32–34]

Cell proliferation assays

Stock cultures were harvested by trypsinization and seeded in triplicate at a density of 1×10^4 per well into 24-well multi-well plates. The final volume of each well was 0.5 ml. After 24 hr, each well received medium only (controls) or medium containing various concentrations of arginine deiminase, SU5416 or DC101. Vascular endothelial cells also received VEGF or bFGF (final concentrations: 20 or 5 ng/ml, respectively). Cells were counted after 3 days with a Coulter particle counter (Beckman Coulter, Krefeld, Germany).

Generation and treatment of experimental neuroblastomas

WAC2 neuroblastoma cells were harvested by trypsinization and injected subcutaneously into the right hind leg of immunedeficient nude mice at a density of 2×10^7 in 0.2 ml of cell culture

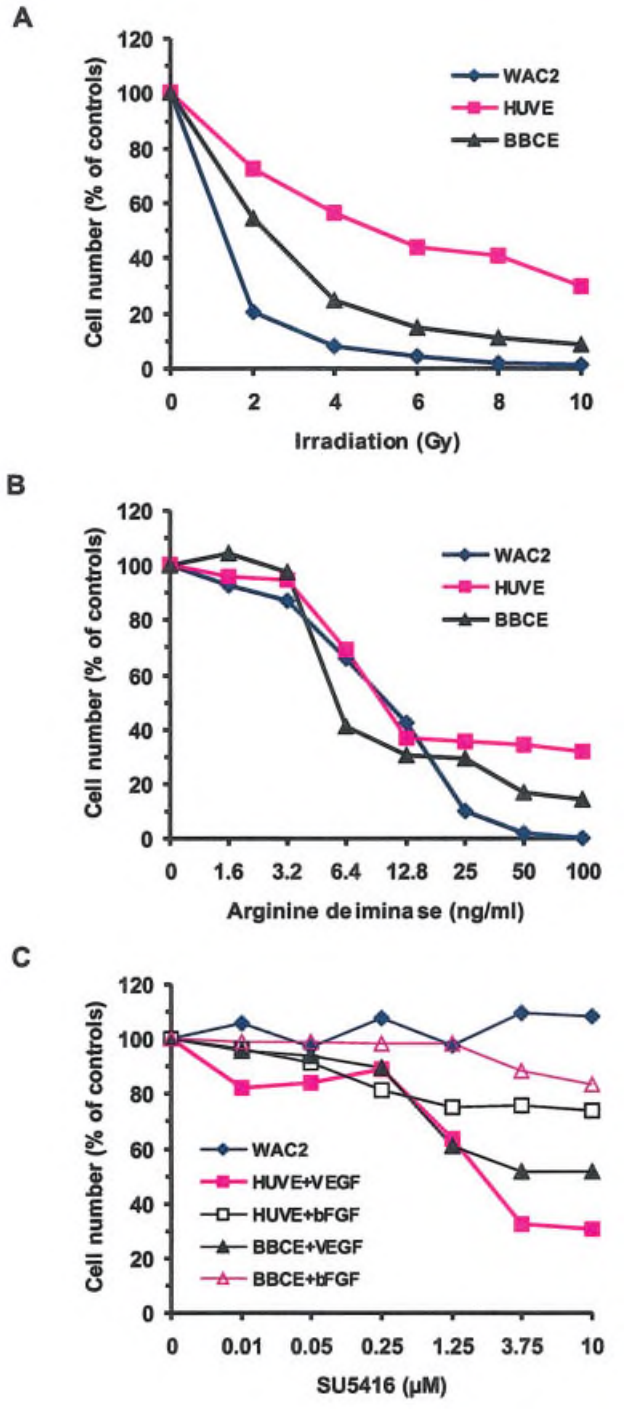

FIGURE 1 – Effect of irradiation (*a*) or the antiangiogenic agents arginine deiminase (*b*) or SU5416 (*c*) on the proliferation of neuroblastoma or vascular endothelial cells *in vitro*. Human WAC2 neuroblastoma cells or vascular endothelial cells derived from bovine brain (BBCE) or human umbilical veins (HUVE) were seeded at densities of 1×10^4 per well and received increasing doses of irradiation, or concentrations of arginine deiminase or SU5416. Vascular endothelial cells also received one addition of VEGF (20 ng/ml) or bFGF (5 ng/ml), respectively. Cells were counted after 3 days as described under Material and Methods. Results are expressed as percentage of cells that had received medium only (controls) and are the averages of triplicate determinations that varied by less than 5% of the means.

TABLE I – SUMMARY OF EFFECT OF ANTIANGIOGENIC AGENTS WITH OR WITHOUT IRRADIATION ON NEUROBLASTOMA GROWTH DELAY *IN VIVO*[1]

Treatment		Growth delay (days; 95% confidence interval) to reach		
Antiangiogenic agent + Irradiation		2-	4-	8-
day 1 to day 5	day 1	fold tumor volume		
PBS (controls)	—	2.8 (2.3–3.4)	4.4 (4.1–4.6)	6.5 (6.2–6.7)
Arginine deiminase	—	3.7 (3.3–4.3)	5.7 (5.4–6.1)[2]	9.9 (9.6–10.3)[2]
DC101	—	3.5 (2.9–4.2)	6.3 (5.7–7.0)[2]	10.7 (10.5–10.9)[2]
SU5416	—	3.7 (2.9–4.2)	n.d.	n.d.
Arginine deiminase + SU5416	—	4.0 (1.6–9.9)	6.3 (6.2–6.4)[2]	8.3 (5.4–12.8)[2]
Arginine deiminase + DC101	—	3.2 (2.6–4.0)	5.6 (4.8–6.6)[2]	8.1 (6.9–9.6)[2]
PBS	6 Gy	2.6 (2.3–3.0)	4.8 (4.3–5.3)	8.9 (8.4–9.4)[2]
Arginine deiminase	6 Gy	4.8 (3.9–5.9)[2,3]	7.4 (6.5–8.4)[2–4]	10.6 (10.1–11.1)[2,3]
DC101	6 Gy	10.6 (8.2–14.3)[2–4]	15.7 (13.0–19.3)[2–4]	19.9 (16.7–23.9)[2–4]
SU5416	6 Gy	7.1 (4.7–10.7)[2–4]	9.2 (5.6–15.1)[2,3]	13.5 (10.3–17.8)[2,3]
PBS	12 Gy	11.0 (9.0–13.9)[2]	22.8 (16.7–32.7)[2]	26.7 (20.2–36.6)[2]
Arginine deiminase	12 Gy	15.2 (12.2–19.5)[2,4]	19.0 (15.7–23.3)[2–3]	31.8 (26.5–38.7)[2,4]
DC101	12 Gy	21.6 (16.6–28.9)[2–4]	25.7 (19.8–34.6)[2–4]	33.3 (25.3–45.7)[2,4]
SU5416	12 Gy	n.d.	n.d.	n.d.

[1]Mice bearing neuroblastomas were randomly divided into groups of 8 animals and received the treatment indicated above. Tumor growth delay was calculated as described in Material and Methods, and expressed as the mean days with 95% confidence interval–[2]$p < 0.05$ for treated mice versus controls,–[3]$p < 0.05$ for combination treated mice versus mice treated by the respective single irradiation (6 or 12 Gy) treatment,–[4]$p < 0.05$ for combination treated mice compared *vs.* mice treated by the respective drug (arginine deiminase, DC101 or SU5416) alone; n.d., not determined.

medium. For all treatment studies, mice were randomly divided into groups of 8 animals. All experiments were started once tumors had reached volumes between 30 and 65 mm^3 (mean: 50 mm^3). Treatment start was defined as day 1.

For irradiation, the tumor-bearing mouse legs were irradiated on day 1 with Photons of 5 MeV applied by a linear accelerator (MEVATRON®, Siemens, Erlangen, Germany) at a dose rate of 2.5 Gy per minute as described previously.[35] The focus isocenter distance was 100 cm with field sizes of 3×2 cm^2 at the isocenter. A total single dose of irradiation delivered was either 6 Gy or 12 Gy. The remainder of the mouse body was shielded from direct irradiation resulting in a whole body dose of less than 8% of the total tumor-absorbed dose.

To examine the effect of antiangiogenic agents on tumor growth, each animal received either arginine deiminase, DC101 or SU5416 at dose of 5, 6.5 or 25 mg/kg in buffer, respectively, once a day by intraperitoneal injection. Control animals received PBS buffer only. Treatments started on day 1 and ended on day 5.

Combined treatments consisted of single irradiation on day 1 plus application of either of the antiangiogenic agents from day 1 to 5.

Quantification of tumor growth and delay

Tumor growth was measured every other day using calipers. Tumor volumes (V) were calculated according to the equation: $V = (a^2 \times b)/2$ where a is the shortest diameter and b is the longest diameter of tumors. Median time to reach 8-fold initial tumor volume was considered as endpoint for growth delay calculations. Median growth delay (GD) was calculated as GD = GD (t) − GD (c) where GD (t) is the median growth time of the individual treatment group (until reaching 2-, 4- or 8-fold tumor volume) and GD (c) is the median growth time of the control group. Data were corrected using the product-limit method of Kaplan-Meier if intercurrent deaths occurred.

Determination of tumor vessel density and perfusion

Tumor-bearing mice received the perfusion marker Hoechst 33342 (15 mg/kg in 50 μl of PBS) by injection through the tail vein and were killed 3 min later. Tumors were removed, frozen and cut into 6 μm sections using a freeze microtome. Sections were air-dried, fixed in acetone, rehydrated with PBS and then incubated with goat anti-type IV collagen (which served as a microvasculature marker) for 45 min at room temperature. After washing with PBS, sections were incubated for 30 min at room temperature with TRITC-labeled donkey anti-goat IgG (which served as a second antibody). Microvasculature and perfusion were detected by scanning each section with a fluorescence microscope by subsequently using ultraviolet light with a 510–560 nm excitation and a 590 nm emission filter and then with a 365 nm excitation and a 420 nm emission filter. The scans resulted in red or blue signals, for vessels or perfusion, respectively.[36] Digital image analysis using software from Imtronic, Inc. (Berlin, Germany) allowed the exact allocation and quantity of red and blue fluorescence signals. Quantitative analysis determined tumor vessel density as vascular area (red) divided by total tumor area and tumor vessel perfusion as proportion of perfused vessels: perfused vessels (blue) in proportion to the total vascular area (red).

Determination of tumor hypoxia

Intratumor oxygen partial pressure (pO_2) was assessed on day 5 by using the Eppendorf histograph (KIMOC 6650, Eppendorf, Hamburg, Germany) as mentioned earlier.[37] The oxygenation status of each individual tumor was described by the fraction of hypoxia pO_2 values below 2.5 or 5 mmHg.

Statistical analysis

Means, standard errors (SE) and 95% confidence interval were calculated by use of the Microsoft Excel SR-2 software package. Comparisons of immunohistochemistry results (vascular density and perfused vessels) were done using the SAS software package (SAS, Inc. Calgary, TX). Differences with a value of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of various treatment modalities on cell growth in vitro

To determine whether irradiation or antiangiogenic agents inhibit the growth of neuroblastoma or vascular endothelial cells, and if so, at what concentrations, we first studied the effect of irradiation or antiangiogenic agents alone on cell proliferation *in vitro*. Irradiation inhibited proliferation of neuroblastoma (WAC2) or vascular endothelial cells derived from bovine brain (BBCE) or human umbilical veins (HUVE) with half-maximal inhibition achieved at approximately 1, 3 or 5 Gy, respectively (Fig. 1*a*). The unspecific angiogenesis inhibitor arginine deiminase inhibited proliferation of these same cells in a potent manner, with half maximal inhibition achieved at concentrations of 5–10 ng/ml, thereby confirming previous data (Fig. 1*b*). As a specific angiogenesis inhibitor, we selected SU5416, which acts by blocking the VEGF pathway in vascular endothelial cells. As expected, SU5416 had

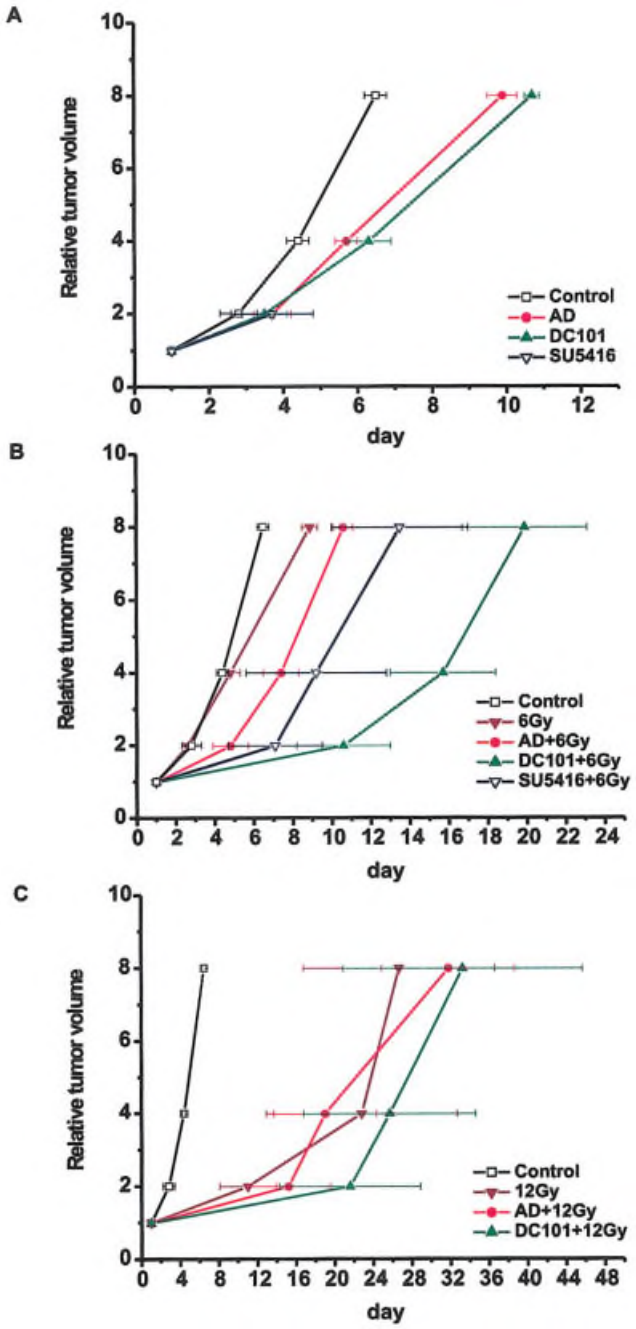

FIGURE 2 – Time course of the effect of antiangiogenic agents alone (*a*) or in combination with doses of 6 Gy (*b*) or 12 Gy (*c*) of irradiation on the growth of experimental human neuroblastomas *in vivo*. Neuroblastomas were generated by subcutaneous injection of WAC2 neuroblastoma cells. Treatment was started once tumors had reached an average volume of 50 mm^3. Each treatment group consisted of 8 animals. Arginine deiminase (AD), DC101 or SU5416 were applied daily from day 1 to day 5 at doses of 5, 6.5 or 25 mg/kg, respectively, while irradiation was applied once on day 1. Controls received PBS only. Tumor volumes were measured as described under Material and Methods. Relative tumor volume was determined as the tumor volume at the indicated day divided by the initial tumor volume on day 1 (30–65 mm^3). Error bars represent SE.

negligible or no effects on the proliferation of the neuroblastoma cell line WAC2 or the proliferation of vascular endothelial cells (BBCE or HUVE) stimulated with the angiogenesis stimulator bFGF. However, it inhibited the proliferation of BBCE or HUVE stimulated with the angiogenesis stimulator VEGF in a potent manner, where half-maximal inhibition was seen at 1–10 μM concentrations (Fig. 1*c*). The other specific angiogenesis inhibitor DC101 was not tested in the *in vitro* setting, since it acts specifically against the mouse VEGFR2 receptor and is known to be inactive in human or bovine cells.

Effect of single treatment modalities on neuroblastoma growth in vivo

We next generated human neuroblastomas by injecting WAC2 cells into immune-deficient nude mice. We then examined the effect of either irradiation or various antiangiogenic agents on the growth of these experimental neuroblastomas. Treatments were started once tumors had reached sizes of 50 mm^3 and their effect documented as the ability to induce growth delay. The nonspecific or specific angiogenesis inhibitors arginine deiminase or SU5416 and DC101 alone or in combination delayed neuroblastoma growth *in vivo* with arginine deiminase and DC101 having significant effects after only 6 days (Table I, Fig. 2*a*). Irradiation of the tumors with 6 Gy led to a significant growth delay after 8 days (Table I, Fig. 2*b*). These data demonstrate that irradiation or angiogenesis inhibitors delay the growth of experimental neuroblastomas with unfavorable properties. Also they demonstrate for the first time that arginine deiminase inhibits neuroblastoma growth *in vivo*.

Effect of combined treatment modalities on neuroblastoma growth in vivo

We next examined whether or not antiangiogenic agents were able to potentiate the growth delay of neuroblastomas irradiated with 6 Gy. In fact, arginine deiminase, SU5416 and DC101 all led to a significant further delay of neuroblastoma growth after only 2 days of treatment, as compared to irradiation only (Fig. 2*b*, Table I). Similar but less significant data were obtained when 12 Gy instead of 6 Gy of irradiation were used (Fig. 2*c*, Table I). From these data, we conclude that antiangiogenic agents potentiate the delay of neuroblastoma growth induced by irradiation. Our data also demonstrate for the first time that arginine deiminase potentiates the inhibitory effect of irradiation on tumor growth.

Mechanisms of in vivo *antitumor effect of various treatment modalities*

The mechanisms that lead to neuroblastoma growth delay induced by antiangiogenic agents in combination with irradiation are unclear, but a reduction of tumor vessel densities appeared as a possibility meriting further investigation.

In fact, as demonstrated in Figure 3*a*, irradiation alone reduced relative vascular density to some extent, and this effect was significantly potentiated by arginine deiminase, DC101 or SU5416.

Interestingly, irradiation alone eventually led to an increase in the proportion of perfused vessels. In contrast, all antiangiogenic agents significantly blocked this effect and led to a net loss of the perfused tumor vessels (Fig. 3*b*).

It is generally believed that antiangiogenic treatment of tumors results in tumor hypoxia and that this may compromise the effect of simultaneous irradiation. We have examined the effect of arginine deiminase, DC101 or irradiation on hypoxia of the experimental neuroblastomas. As demonstrated in Table II, arginine deiminase alone did not augment tumor hypoxia, but DC101 or irradiation alone did so. In contrast, neither arginine deiminase nor DC101 augmented tumor hypoxia induced by irradiation, when combined with irradiation.

DISCUSSION

Neuroblastomas of advanced stages that contain amplified *N-myc* have unfavorable properties, including the ability for rapid proliferation *in vitro* and *in vivo* and the potential for autonomous growth involving extensive angiogenesis. Such unfavorable neu-

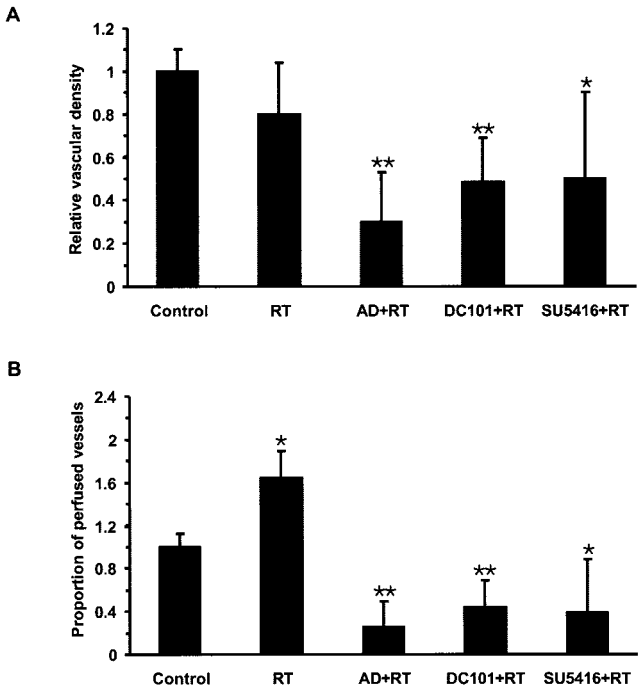

FIGURE 3 – Effect of irradiation alone or in combination with antiangiogenic agents on the densities (*a*) or perfusion (*b*) of neuroblastoma tumor vessels. Generation of neuroblastomas and treatment strategies were as described under Material and Methods and in the legend to Figure 2. The irradiation (RT) dose was 6 Gy in all experiments. Neuroblastomas were analyzed after 5 days of treatment initiation. Relative vascular densities and the proportion of perfused vessels determined as outlined under Material and Methods. Error bars represent SE. $*p < 0.05$; $**p < 0.01$ for treated mice compared to controls.

TABLE II – EFFECT OF ANTIANGIOGENIC AGENTS WITH OR WITHOUT IRRADIATION ON INTRATUMOR OXYGEN PARTIAL PRESSURE[1]

Treatment	Hypoxic fraction (%) with pO_2	
	< 2.5 mmHg	< 5.0 mmHg
PBS (controls)	52.8	61.1
Arginine deiminase	50.0	61.1
DC101	80.6[2]	94.4[2]
Irradiation	79.2[2]	83.3[2]
Arginine deiminase + irradiation	72.9[2]	77.1[2]
DC101 + irradiation	70.8[2]	79.2[2]

[1]Mice with average tumor volumes of 50 mm^3 received arginine deiminase and DC101 daily from day 1 to 5 at doses of 5 or 6.5 mg/kg, respectively, while irradiation was applied as a single dose of 6 Gy on day 1. Controls received PBS only. Treatment groups consisted of 8 animals each. Hypoxia was determined by the fraction of intratumor oxygen partial pressure (pO_2) values below 2.5 or 5 mmHg after 5 days of treatment initiation as described in Material and Methods.– [2]$p < 0.05$ for treated mice compared with controls.

roblastomas often relapse upon therapy. We have previously shown that arginine deiminase strongly inhibits the proliferation of cultured neuroblastoma and vascular endothelial cells by arresting cell cycle and inducing apoptosis.[29] Arginine deiminase inhibits proliferation of actively diving, but not quiescent, cells *in vitro*, and had no detectable side effects *in vivo* over the time period studied (data not shown). These properties suggested a lack of general cytotoxicity and its clinical potential as an antitumoral and antiangiogenic drug for the treatment of unfavorable neuroblastomas, possibly in combination with other modalities.

Here, we have demonstrated that arginine deiminase strongly inhibits tumor growth *in vivo*. This involves arginine deiminase-mediated increase in the fraction of G2-phase cells and the corresponding decrease in active S-phase cells (*i.e.*, cells with S-phase DNA content incorporating BrdU) *in vivo* (data not shown), thus corroborating our earlier findings obtained i*n vitro*.[29] Arginine deiminase may mediate cell cycle arrest by a direct effect on the tumor cells but also by indirect means, for example, by compromising angiogenic mechanisms. In fact, serum VEGF levels in animals treated with arginine deiminase or other antiangiogenic agents including DC101 and SU5416 were significantly reduced as compared to control animals (data not shown), suggesting that inhibition of VEGF signaling in the tumors may, at least partially, be involved in their mechanisms of action. DC101 is known to inhibit growth of cells derived from favorable neuroblastoma alone or in combination with chemotherapy.[38] Here, we have demonstrated that the antiangiogenic effect of DC101 on neuroblastomas can also be potentiated by irradiation.

Irradiation of the neuroblastomas with 6 Gy had little, if any, effect on tumor vessel densities but increased the relative proportion of perfused tumor vessels. However, when combined with arginine deiminase or other antiangiogenic agents, it reduced both relative vessel densities and vessel perfusion. In principle, this should lead to tumor hypoxia. However, under our experimental conditions, the combination of arginine deiminase or DC101 with irradiation did not augment tumor hypoxia induced with irradiation alone. This surprising finding is in contrast to those obtained by others that antiangiogenic treatment may actually reduce the efficacy of irradiation by reducing oxygenation of tumor tissue,[39,40] for example, with TNP-470.[41] However, tumor histology and treatment periods and intensities all play a significant role in the qualitative and quantitative tumor response. Thus, our data may be the results of the tumor histology and the experimental condition chosen. Nevertheless, they suggest that in neuroblastoma, the combination of antiangiogenesis and irradiation may provide a strategy with clinical potential. Combination therapy may offer the additional advantage of potentiating tumor response while reducing side effects that would be observed with high-dose single modality treatment approaches.

In summary, we have demonstrated here for the first time that arginine deiminase 1) inhibits neuroblastoma growth *in vivo* and 2) potentiates irradiation-induced inhibition of neuroblastoma growth. The cells chosen here had aggressive properties that closely resembled a clinically unfavorable set of neuroblastomas with a strong tendency to relapse. Our data suggest that arginine deiminase, alone or in combination with irradiation, may offer a means to treat neuroblastomas with otherwise poor prognosis.

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