Continuous Versus Intermittent Administration of Human Endostatin in Xenografted Human Neuroblastoma

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Purpose: The authors examined whether recombinant human endostatin (rhEndostatin), an antiangiogenic agent, is effective against a human neuroblastoma cell line (designated TNB9) using a human neuroblastoma xenograft model and investigated whether continuous infusion is more effective than intermittent administration.

Methods: In the first experiment, when tumors on the back of nude mice reached a weight of 90 to 95 mg, rhEndostatin, 10 mg/kg/d mouse weight, was administered subcutaneously to the mice (n = 5) every day for 10 consecutive days. In the second experiment, the same daily dose of rhEndostatin was administered continuously to the TNB9-bearing mice (n = 6) via subcutaneous infusion pumps for 3 consecutive days with total dose being 30% of that in the first experiment. Nestin and factor VIII expression levels were studied immunohistochemically to elucidate whether histologic evidence of the effects of rhEndostatin was present on day 4 in the second experiment.

Results: In the first experiment, relative tumor weight in treated mice (n = 5) was significantly less than that in controls (n = 12) on day 2 only after treatment initiation ($P < .05$). The maximum inhibition rate (MIR) of TNB9 xenograft growth by rhEndostatin was 46.4%, indicating lack of efficacy. In the second experiment, the effects of rhEndostatin were much more marked than those in the first experiment, with an MIR of 60.7%. The mean relative tumor weight in the treated group (n = 6) in the second experiment was significantly less than that in controls (n = 10) on days 2, 4, and 6 ($P < .01$) as well as on days 8 and 10 ($P < .05$). Nestin staining in the endothelium of control tumors (n = 2) was marked, whereas it showed a loss of fibrillar structure in rhEndostatin-treated tumors (n = 2). The number of vessels immunostained with antifactor VIII antibody was markedly reduced in tumors (n = 2) from rhEndostatin-treated mice compared with that in tumors from control animals (n = 2).

Conclusions: Continuous administration of rhEndostatin resulted in more significant tumor regression than intermittent administration of the agent in the same model. This indicates that rhEndostatin, if administered in continuous fashion, could become an effective agent for treating patients with neuroblastoma in the future.

INDEX WORDS: Neuroblastoma, antiangiogenic drug, human endostatin, osmotic infusion pump, nude mouse, nestin, factor VIII.

NEUROBLASTOMA is the most common solid tumor in children and is highly malignant in patients older than 12 months with distant metastases or with amplification of the *MYCN* oncogene.[1,2] Despite recent multimodal treatment regimens that include high-dose chemotherapy, irradiation, surgery, and blood stem cell transplantation, less than 30% of patients older than 12 months of age with stage 4 disease can be expected to survive.[1-3] To improve the clinical results further, some new modalities or new antitumor agents must be added to current treatment regimens for neuroblastoma.

In addition to chemotherapeutic agents, the effects of several antiangiogenic agents such as endostatin, angiostatin, TNP-470, antivascular endothelial growth factor (VEGF) antibodies, epigallocatechin gallate, and others have been studied to determine whether these agents lead to tumor regression in vivo systems.[1-17] There is a considerable body of direct evidence that tumor growth is angiogenesis dependent.[10,18,19]

Among antiangiogenic substances, Endostatin, the 20-kDa C-terminal fragment of collagen XVIII, has been shown previously to inhibit the growth of various xenotransplanted human tumors and of experimental tumors in rodents.[4-8] Recently, the gene for recombinant human endostatin (rhEndostatin) was cloned and expressed,[20] and rhEndostatin is being used in phase I studies on adult

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cancer patients. In these first phase I clinical trials of endostatin as an antiangiogenic therapy for cancer,[21] the protein was administered as an intravenous (iv) bolus for approximately 20 to 30 minutes each day. This protocol was based on experimental studies[4,9] in which animals were treated by subcutaneous (sc) bolus once a day. Because it was not clear in the previous studies whether this schedule could be maximized further, continuous administration has been explored, and there have been 2 recent studies[22,23] that showed that continuous release of human endostatin is more effective than intermittent administration in human pancreatic cancer and glioma cell lines. In this article, we report on the results of our experiment to determine whether continuous or intermittent administration of rhEndostatin is effective against xenografted human neuroblastoma using a human neuroblastoma xenograft designated TNB9.[24] As a histologic indication of angiogenesis inhibition, immunostaining for an endothelial precursor cell marker, nestin,[25] and a mature endothelial cell marker, factor VIII,[26] was performed.

MATERIALS AND METHODS

The human neuroblastoma xenograft, TNB9, originally derived from stage 4 abdominal neuroblastoma with *MYCN* amplification in a 15-month-old boy, was used.[24] Our preparatory studies confirmed that the original biological characteristics, in particular, the *MYCN* amplification status and chromosome findings, were retained after serial transplantations.

A small portion of minced tumor about 2.5 mm in diameter was inoculated subcutaneously with a trochar into the back of 6-week-old female BALB/C-*nu/nu* athymic mice. Treatment was initiated when the tumors had reached a weight of 90 to 95 mg. Tumor-bearing mice were divided randomly into groups of 5 and 12 animals each, or 8 and 12 animals each. rhEndostatin was purchased from Calbiochem (San Diego, CA). The efficacy of rhEndostatin was confirmed as in a preliminary study.[27] In the first experiment, rhEndostatin, 10 mg/kg/d mouse weight (MW)/sc was injected into 5 tumor-bearing nude mice for 10 consecutive days based on the successful results of O'Reilly et al in mouse model,[4] and control mice (n = 12) received saline, 10 mL/kg MW/d sc for 10 consecutive days. In the second experiment, the same daily dose of rhEndostatin as in the first experiment was administered to the mice (n = 8) continuously for the first 3 days with a set of 3 osmotic pumps (Alzet; Alza Corporation, Palo Alto, CA) implanted in each mouse (Fig 1). Therefore, total dose of rhEndostain in the second experiment was 30% of that in the first experiment. The 3 pumps in control animals (n = 12) contained 0.9N NaCl.

The size of the tumor and body weight of the experimental and control mice were recorded every other day from the start of administration of rhEndostatin until day 16. Two perpendicular tumor diameters were measured with a slide caliper. Tumor weights were assumed according to the formula: weight (mg) = length (mm) × width $(mm)^2 \times 0.5$, where the length and the width are the largest and smallest perpendicular diameters, respectively. The tumor weights in the experimental and control groups were compared every other day, and statistical evaluation was performed with the nonparametric Mann-Whitney U test. The results also were evaluated according to the protocol of Battelle Columbus Laboratories.[28,29] The effects of treatment were expressed as the maximum inhibition rate (MIR), $(1 - T_{RW}/C_{RW}) \times 100$ (%), where T_{RW} is the mean relative tumor weight compared with the initial weight of the treatment group, and C_{RW} is that of the control group. When the results were evaluated using the Battelle Columbus laboratories protocol, agents were considered to be "highly effective" when regression of tumor growth ($T_{RW} < 1.0$) was seen, "effective" when retardation of tumor growth (MIR $\geq$ 58%) was observed, and "ineffective" when the MIR was less than 58%.[24,28,29]

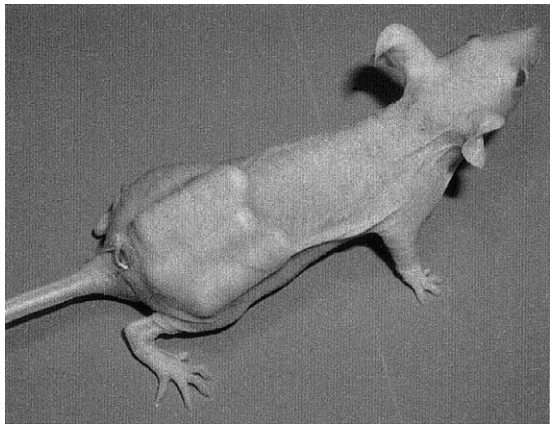

Fig 1. Three osmotic pumps were implanted in each mouse in the second experiment.

In the first experiment, 2 mice in each group were killed on day 16 by carbon dioxide asphyxiation, and tumor samples were fixed in 10% formalin and embedded in paraffin for histologic study with H & E staining. From 3,019 to 5,000 cells per specimen were counted in determining mitosis-karyorrhexis index (MKI) and number of mitosis.

In the second experiment, 2 mice in each group were killed on day 4 similarly, and tumor samples were fixed and embedded in the same fashion for histologic and immunohistochemical studies. Sections 4 μm thick then were stained with H & E for conventional histologic assessment, and with antinestin[25] and antifactor VIII[26] antibodies.

The antibody to human nestin was generated in rabbits using the synthetic oligopeptide covering 17 C-terminal amino acids: 1602-KFTQREGDRESWSSGED-1618, as described elsewhere.[30] The tissue sections and bovine aortic endothelial cells (control) were first incubated with the primary antibody to nestin at a dilution of 1:5000. An LSAB2/HRP staining kit (DAKO Corporation, Santa Barbara, CA) was used as the secondary reaction system. The procedure consisted of a secondary antibody reaction followed by an enzyme reaction with a horseradish peroxidase-labeled streptavidin system. The nucleus was counterstained blue with 4,6-diamino-2-phenylindole.[25]

After processing of slides with 3% H_2O_2, antifactor VIII–related antigen antibody (DAKO Corporation) at a 1:80 dilution was applied dropwise on the slides in a wet chamber. Either $LSAB^+$ kit or EnVision kit (DAKO) was used. Then the slides were rinsed with phosphate-buffered saline, and a secondary antibody (1:100) was applied to the slides for 60 minutes in the wet chamber. Thereafter, substrate-chromogen solution (1:50) was added dropwise for 10 to 20 minutes in the wet chamber. Hematoxylin was used for counterstaining.[26]

RESULTS

The assumed tumor weights from day 0 to 10 are shown in Table 1. In the first experiment, the tumor weights were significantly less than those in the control group on day 2, but from day 4 to day 10, the tumor weights in the rhEndostatin and control groups did not differ significantly (Fig 2). The MIR of rhEndostatin in the TNB9 model was 46.4%. None of the 5 mice in the

Table 1. Changes in Relative Tumor Weight in Experimental and Control Groups

Days	Relative Tumor Weight in rhEndostatin-Treated Mice	Relative Tumor Weight in Controls	*P* Values
First experiment*			
2	0.97 ± 0.24	1.81 ± 0.70	.0112
4	1.32 ± 0.17	2.17 ± 1.01	.0509
6	2.18 ± 0.43	3.74 ± 1.62	.0911
8	4.71 ± 0.59	5.74 ± 1.80	.2249
10	7.73 ± 0.70	9.08 ± 3.30	.5966
Second experiment†			
2	0.90 ± 0.24	2.29 ± 0.68	.0030
4	1.58 ± 0.73	3.18 ± 0.64	.0045
6	2.06 ± 0.80	4.58 ± 1.08	.0030
8	3.97 ± 1.59	7.08 ± 1.80	.0280
10	5.34 ± 1.82	9.50 ± 2.56	.0237

*rhEndostatin-treated mice (n = 5); Control (n = 12).
†rhEndostatin-treated mice (n = 6); Control (n = 10).

rhEndostatin-treated group or the 12 mice in the control group died during the experimental period. No decrease in body weight was observed in any of the mice in either group during experiment.

In the second experiment, the tumor weights in the rhEndostatin-treated group were significantly less than those in the control group on day 2 through day 10 (Fig 2). The MIR of rhEndostatin was 60.7%, indicating that it was "effective" according to the Battelle Columbus Laboratories protocol, and tumor growth regression occurred ($T_{RW} < 1.0$), indicating that rhEndostatin was "highly effective." None of the 6 mice treated with continuous rhEndostatin infusion and none of the 10 mice in the control group died during the experimental period. The set of 3 osmotic pumps was retained successfully in the subcutaneous tissue of the treated and control animals throughout the experiment. No decrease in body weight was observed in either group during the second experiment.

In the first experiment, the tumors in the controls on day 16 had few necrotic areas, and there was little increase in the number of necrotic areas in tumors on the rhEndstatin-treated mice.[27] The mean mitosis-karyorrhexis index (MKI) was similar in each of the subgroups analyzed. There was no significant difference in the percentage of necrosis detected in the tumors from the animals treated with rhEndostatin versus those from the saline-treated controls. Similarly, the numbers of vessels counted in the treated tumors did not differ significantly from that observed in the control tumors.[27]

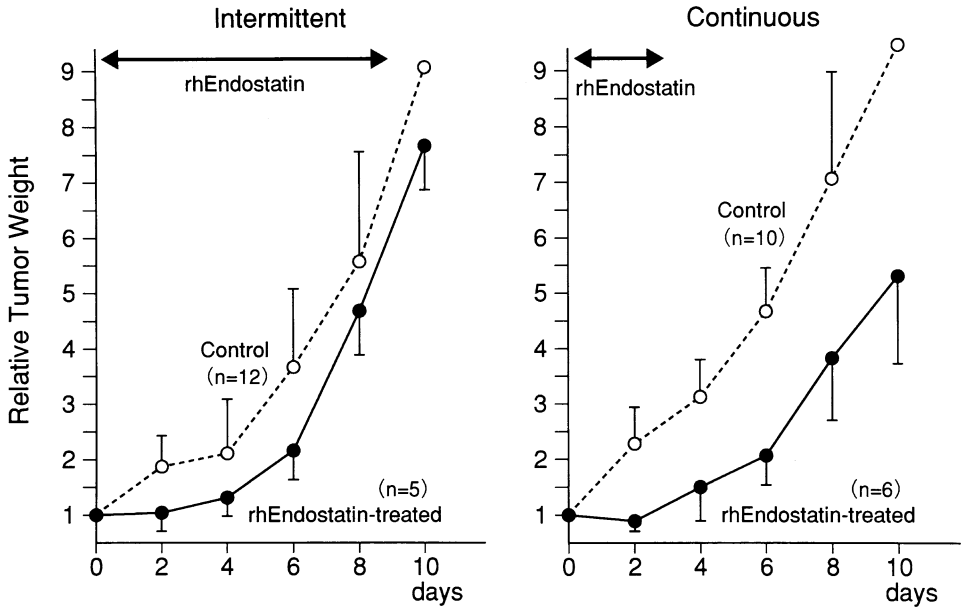

Fig 2. Tumor weight curve for the group of mice treated with intermittent and continuous administration of rhEndostatin. Arrow and bar, administration of agent; ○, control; ●, rhEndostatin-treated (10 mg/kg MW/d, sc). Bars indicate SEM.

Table 2. Histopathologic Changes in Tumors on Mice in the Experimental and Control Groups (Day 4 of the Second Experiment, H & E)

Samples	Tumor 5*	Tumor 6*	Tumor E†	Tumor F†
Necrosis (area %)	1%	5-6%	0%	0%
Mitosis-karyorrhexis index (MKI)‡	3.35%	3.42%	5.30%	6.02%
Mitosis§	2.16%	2.32%	1.66%	2.50%

*Control tumors.

†rhEndostatin-treated tumors.

‡Number of cells showing mitosis-karyorrhexis/number of cells counted.

§Number of cells showing mitosis/number of cells counted.

In the second experiment, however, HE sections showed an increase in the MKI index in rhEndostatin-treated tumors on day 4 when compared with control tumors (Table 2). Immunostaining with antinestin antibody[25] and antifactor VIII antibody[26] showed marked histologic differences between the treated and control groups on day 4. Nestin staining in the endothelium of control tumors (n = 2) was marked mainly in the vasculature of the surrounding tissue but also in the tumor, whereas it was fainter and showed a loss of intact fibrillar structure of the peritumoral vasculature in rhEndostatin-treated tumors (n = 2; Fig 3). The number of the intratumoral vessels clearly immunostained with antifactor VIII antibody[26] was markedly reduced in tumors (n = 2) from rhEndostatin-treated mice compared with that in tumors from control animals (n = 2; Fig 4; Table 3).

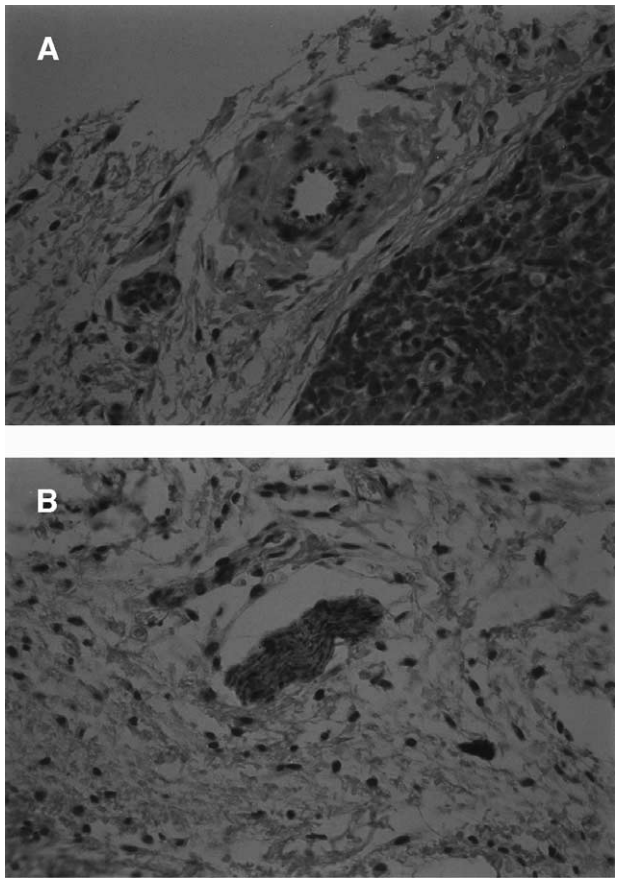

Fig 3. Immunostaining for nestin of the peritumoral vascular structure of human neuroblastoma xenograft TNB9 in controls and in the group treated with rhEndostatin in the second experiment. Specimens were obtained on day 4 and stained with antinestin antibody.[25] (A) Marked immunostaining for nestin was observed in the endothelium of intact peritumoral vasculature of control tumors. (B) rhEndostatin-treated tumor. Nestin staining was fainter and showed a loss of intact fibrillar structure of the peritumoral vasculature in the rhEndostatin-treated tumor. (Original magnification ×400.)

DISCUSSION

The treatment of advanced neuroblastoma has improved significantly in recent years,[1-3] but the overall survival rate of patients with advanced *MYCN*-amplified neuroblastoma remain only 28.8% at 66 months as reported by Kawa et al.[3] To improve the clinical results further, the introduction of new chemotherapeutic agents into clinical protocols is essential, and the authors' group has conducted a number of experimental studies during the past 18 years.[24,31] The neuroblastoma xenograft TNB9 has been used most frequently in our previous experiments that showed the effectiveness of various chemotherapeutic agents such as cyclophosphamide, cisplatin, dacarbazine, melphalan, carboplatin, irinotecan, and others and the ineffectiveness of vincristine, cytosine arabinoside, fotemustine, and busulfan.[24,32] TNB9, originally derived from an abdominal neuroblastoma in a 15-month-old boy, was established as a xenograft and has been maintained by serial transplantation into nude mice. TNB9 has chromosomal abnormalities pertinent to neuroblastoma as well as *MYCN* plus *DDXI* amplifications and is associated with a short doubling time.[24]

Among the agents currently being investigated in clinical trial protocols, antiangiogeneic agents are expected to contribute to the treatment of advanced neuroblastoma, particularly because of the higher vascular index of this tumor in advanced stages.[18] Among several antiangiogenic agents such as TNP-470, anti-VEGF antibody, angiostatin, endostatin, and epigallocatechin gallaste, O'Reilly et al[4] clearly showed the potent antitumor effects of mouse endostatin against mouse tumors such as Lewis lung carcinoma, T241 fibrosarcoma, B16F10 melanoma, and EOMA hemangioendothelioma. They also observed that recombinant mouse endostatin dose dependently inhibited Lewis lung carcinoma primary tumors. Potent tumor growth inhibition was seen when the tumor-bearing mice were treated with doses of 10 and 20 mg/kg MW/d sc for 10 consecutive days, whereas no antitumor effects were observed when the mice were given a dose of 2.5 mg/kg MW/d for 10 days.[4] Subsequently, Perletti et al[33] showed the antitumor activity of recombinant rat endostatin against carcinogen-induced

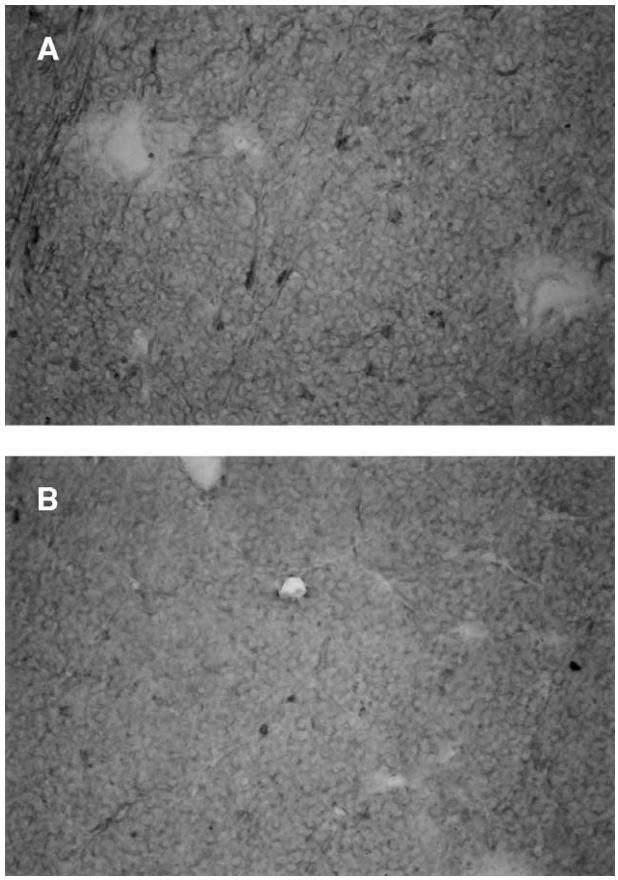

Fig 4. Immunostaining for factor VIII of the human neuroblastoma xenograft TNB9 in controls and in the group treated with rhEndostatin in the second experiment. Specimens were obtained on day 4 and stained with anti-factor VIII antibody.[26] An LSAB$^+$ kit was used. (A) Control tumor (B) rhEndostatin-treated tumor. The number of vessels immunostained with anti-factor VIII antibody was reduced markedly in tumors from rhEndostatin-treated animals (see also Table 3). (Original magnification ×200.)

rat primary mammary tumors.[33] After those successful experiments, the gene for recombinant human endostatin (rhEndostatin) was cloned and expressed.[20,34] and the recombinant form has been manufactured by EntreMed Inc. (Rockville, MD) under the guidance of Folkman for use in phase I trials in adult cancer patients.[21]

In the current experiments, a significant difference in tumor size was observed in both treatment regimens, especially in the second experiment (Fig 2). This might appear to be an extremely quick onset of action for rhEndstatin, but such phenomenon was already reported by Kisker et al[22] who showed a much significant difference in tumor size in the first and second days of experiment between controls and the group treated with rhEndostatin 20 mg/kg/d by intraperitoneally located osmotic pump.

Decreased vascularization has been noted in tumors treated successfully with antiangiogenic agents.[35] Nestin is one of intermediate filaments, together with vimentin and gial fibrillary acidic protein, and is abundantly detected in neuroepithelial stem/progenitor cells in the growing central nervous system in the embryonic stage and in the angiogenic vascular structure of brain tumors but not in that of the mature neuronal tissues.[25,36] Nestin mRNA is approximately 6.2 kilobases long, and its gene contains 3 introns. Interestingly, neuroepithelium-specific nestin is driven by the second intron of the nestin gene, whereas muscle precursor-specific expression is driven by the first intron.[25,37] Sugawara et al[25] showed immunostaining for nestin also in all 4 samples of hemangiblastomas and postulated that nestin is not only a marker for neuroepithelial stem cells and glioma cells but also for proliferating endothelial cells during rapid growth. Similarly, the presence of factor VIII clearly shows microvessel density and distribution in breast carcinoma and other neoplasms.[26,38-40] Both nestin and factor VIII therefore are regarded as markers of vascularization in antiangiogenic experiments. Decreased vascularization was seen clearly in the second experiment by immunostaining with antinestin and factor VIII antibodies. However, the number of samples in MKI and mitosis studies[41] was too small to draw a conclusion whether effects of decrease in vascularization might lead to an increase in MKI (Table 2).

After extensive investigations, Kisker et al[22] found significant antitumor effects with the continuous administration of rhEndostatin in human pancreatic carcinoma in vivo. The current experiments were analogous to those of Kisker et al,[22] and we confirmed the merits of continuous administration of rhEndostatin in an in vivo model of human neuroblastoma. Joki et al[23] also observed marked antihuman glioma effects of human endostatin with continuous release of the protein from microencapsulated-engineered cells transfected with a rhEndostatin expression vector. In addition to endostatin, Cohn et al[42] showed marked antitumor effects with continuous ad-

Table 3. Number of Vessels in Tumors on Day 4 of the Second Experiment, Immunostained with Antifactor VIII Antibody

Samples	Tumor 5*	Tumor 6*	Tumor E†	Tumor F†
1	13	10	7	10
2	10	21	7	4
3	15	12	11	4
4	12	16	10	8
5	10	10	15	6
6	12	11	4	9
7	6	19	5	9
8	12	21	5	5
9	3	8	8	7
10	11	21	3	8
Mean	10.4	14.9	7.5	7.0
SD	3.50	5.25	3.66	2.16

*Control tumors.
†rhEndostatin-treated tumors.

ministration of an angiogenesis inhibitor derived from Schwann cells.

It has been reported that antiangiogenic agents are effective when the tumor burden is minimal.[13] Previous experimental results of the authors[27] also showed that rhEndostatin was effective only when the tumor mass was small, suggesting that further clinical and experimental studies be performed using continuous injection at the time of minimal tumor burden. Because changes in body weight in the experimental group were minimal, a much higher dose could be used if the agent was available in large quantity. Continuous administration of antiangiogenic agents could also be achieved using gene therapy technique in future.[43]

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