Inhibition of Angiogenesis Induces Chromaffin Differentiation and Apoptosis in Neuroblastoma

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Inhibition of angiogenesis has been shown to reduce tumor growth, metastasis, and tumor microvascular density in experimental models. To these effects we would now like to add induction of differentiation, based on biological analysis of xenografted human neuroblastoma (SH-SY5Y, WAG rnu/rnu) treated with the angiogenesis inhibitor TNP-470. Treatment with TNP-470 (10 mg/kg s.c., $n = 15$) reduced the tumor growth by 66% and stereological vascular parameters (L_v , V_v , S_v) by 36–45%. The tumor cell apoptotic fraction increased more than threefold, resulting in a decrease in viable tumor cells by 33%. In contrast, the mean vascular diameter (29 μ m) and the mean tumor cell proliferative index (49%) were unaffected. TNP-470-treated tumors exhibited striking chromaffin differentiation of neuroblastoma cells, observed as increased expression of insulin-like growth factor II gene (+ 88%), tyrosine hydroxylase (+ 96%), chromogranin A, and cellular processes. Statistical analysis revealed an inverse correlation between differentiation and angiogenesis. It is suggested that by inhibiting angiogenesis, TNP-470 induces metabolic stress, resulting in chromaffin differentiation and apoptosis in neuroblastoma. Such agonal differentiation may be the link between angiostatic therapy and tumor cell apoptosis. (*Am J Pathol* 1999, 154:395–403)

Angiogenesis is a prerequisite for the growth and metastasis of tumors.^{1,2} In several animal experimental models, tumor growth and metastasis can be reduced or inhibited by administration of an angiostatic preparation as single therapy.^{3–5} Moreover, inhibition of angiogenesis has been shown to potentiate the effects of cytotoxic drugs and radiotherapy.^{6,7} The angiogenesis inhibitor TNP-470, an analogue of fumagillin⁸ with well characterized angiostatic activity, is presently in phase III clinical trials. TNP-470 acts by inhibiting endothelial cell proliferation,^{8–10} resulting in a reduction of tumor vascular density. Angio-

static treatment has also been shown to reduce the population of viable tumor cells without exerting a direct cytotoxic effect on tumor cells. This is achieved not through a decrease in tumor cell proliferation, but rather through an increase in tumor cell apoptosis.^{11,12} To these known effects of angiostatic therapy—reduced vascular density, increased tumor cell apoptosis, potentiation of cytotoxic drugs and radiation—we would now like to add induction of differentiation of tumor cells.

Neuroblastoma is an embryonal cancer derived from immature sympathetic cells of any part of the central nervous system.¹³ It is manifested in infancy and early childhood and has extraordinary clinical and biological heterogeneity. In about half of the patients, particularly those over 1 year of age with advanced stages of the disease, the tumor will progress despite intensive therapy. In some patients, especially infants, the tumor may regress spontaneously, whereas in other patients it may differentiate into a benign ganglioneuroma after no or minimal therapy.¹⁴ Traditionally such differentiation has been viewed as evidence that the tumor exhibits a sympathetic neuronal phenotype. Recently, however, it has been found that chromaffin differentiation along a fetal-specific extra-adrenal lineage is a common phenomenon in neuroblastoma areas with focal apoptosis.¹⁵ A specific and useful marker for this type of differentiation is expression of the gene encoding insulin-like growth factor II (*IGF2*), as shown by *in situ* hybridization (ISH).

Neuroblastomas grow rapidly, often give rise to metastases, and are highly vascularized. Angiostatic treatment should thus be feasible. We have developed a new animal experimental model for human neuroblastoma,¹⁶ based on a human neuroblastoma cell line SH-SY5Y¹⁷ xenotransplanted to the subcutaneous tissue of nude rats (WAG rnu/rnu), to evaluate the effects of angiogenesis inhibitors. Treatment with such an inhibitor, TNP-470, resulted in a 66% reduction of neuroblastoma growth after 12 days of treatment (six injections).¹⁶

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The mechanisms underlying this growth reduction were analyzed in this study and addressed by a panel of complementary techniques: unbiased stereological quantification of tumor angiogenesis and vascular casts of tumor microcirculation; specific plasma chromogranin A (CgA) radioimmunoassay, CgA immunohistochemistry (IHC), *IGF2* ISH, and tyrosine hydroxylase (TH) IHC for quantification of tumor differentiation; Ki 67 IHC and terminal deoxynucleotidyl transferase nick end labeling (TUNEL) for quantification of tumor cell proliferation and apoptosis, respectively.

Materials and Methods

Neuroblastoma Cells

The adrenergic neuroblastoma cell line SH-SY5Y¹⁷ was kindly provided by Dr. June Biedler of The Memorial Sloan-Kettering Cancer Center (New York). The cells were grown at 37°C in Eagle's minimum essential medium (Labsystems, Stockholm, Sweden), supplemented with 10% fetal calf serum (Sigma, St Louis, MO), 1 mmol/L L-glutamine, penicillin (100 IU/ml), and streptomycin (50 µg/ml) in a humidified 95% air/5% CO₂ atmosphere. Details of the preparation procedure are given by Wassberg et al.¹⁶ The final concentration was adjusted to 100×10^6 cells/ml and the suspension was placed on ice.

In Vitro Effects of TNP-470

Differentiation of neuroblastoma cells *in vitro* were analyzed using 4-chamber slides (Nunc Lab-Tek, Nunc International, Roskilde, Denmark). Cells were plated and incubated with different concentrations of TNP-470 (0, 10, 100, and 1000 ng/ml) in each well. After 3 days the chamber was removed and the cells fixed in 4% paraformaldehyde for 20 minutes, and then washed in phosphate-buffered saline (PBS) with a few drops of 1 mol/L glycine for 20 minutes. The slides were dried in air, then immediately frozen at -20°C, until ISH or IHC was performed.

Animal Experimental Model

Twenty-eight nude rats (WAG rnu/rnu; Animal Department, Biomedical Centre, Uppsala University), 14 males and 14 females, were used for xenografting at the age of 7–10 weeks (body weight, 102–178 g). We used nude rats because their size is suitable for perfusion fixation and vascular casting. Details of the animal experimental procedure are given by Wassberg et al.¹⁶ Briefly, the animals were injected with 20×10^6 cells suspended in 0.2 ml culture medium, s.c. on the lateral side of each hind leg, using a 23-gauge cannula. Tumor volume was measured with a caliper every other day. To permit accurate measurements, the animals were anesthetized with 2% halothane (ISC Chemicals, Avonmouth, UK) supplemented with 50% N₂O in oxygen. Tumor volume was calculated by the formula $0.44 \times \text{length} \times \text{width} \times \text{width}$.¹⁶

Treatment with TNP-470

TNP-470⁸ was a kind gift of Takeda Chemical Industries (Osaka, Japan). Immediately before treatment, TNP-470 was suspended in 1% ethanol and 5% gum arabic in saline to the appropriate concentration. When a tumor in an animal had reached a volume of 0.3 ml (designated day 0), the animal was randomized to one of two groups. One group received injections of TNP-470 and the other (control) group received no treatment. If both tumors in an animal reached a volume of 0.3 ml on the same day, both tumors were followed; if not, only the largest one was followed. Twelve animals (15 tumors), 6 males and 6 females, were treated for 12 to 17 days with TNP-470. This was injected s.c. once every other day in the neck, in a dose of 10 mg/kg body weight. This dose was chosen since a dosage of 20 or 30 mg/kg results in a rapid loss of body weight by more than 25% in these nude rats.¹⁶ The site of injection was changed each time to avoid skin erosion. Sixteen animals (18 tumors), 8 males and 8 females, killed at 12 to 18 days, served as controls.

Perfusion Fixation and Vascular Casts

The animals were anesthetized by an i.p. injection of sodium barbital (60 mg/kg body weight, Mebumal, ACO, Stockholm, Sweden) and subjected to perfusion fixation or vascular casting on days 26–37 after xenotransplantation. The procedure is presented in detail elsewhere.¹⁸ Briefly, a 16-gauge intravenous cannula (Viggo, Helsingborg, Sweden) was inserted in the thoracic aorta and the animal was perfused with either 4% paraformaldehyde in Millonig's phosphate buffer, pH 7.4, 37°C, for IHC and ISH, or 2.5% glutaraldehyde in PBS, pH 7.4, 37°C, for transmission electron microscopy, or methyl methacrylate resin of a viscosity similar to that of rat's blood for scanning electron microscopy of vascular casts. All perfusates were infused at a pressure of 100–140 mm Hg, as monitored in the left femoral artery.

Specimen Preparation

Specimens perfusion-fixed in formaldehyde were dehydrated and embedded in paraffin. Sections were cut at 4 to 5 µm from the geometrical cross-section of the tumor and put on diaminoalkyl-silane-treated glass slides¹⁹ and deparaffinated before ISH, IHC, or TUNEL. One section from each tumor was routinely stained with hematoxylin and eosin.

In Situ Hybridization

The occurrence of extra-adrenal chromaffin differentiation was determined by analysis of the *IGF2* expression.¹⁵ A³⁵S-labeled antisense riboprobe used for ISH analysis was made from a 680-bp cDNA fragment spanning the coding region in exon 9 of *IGF2* cloned into pGem-3 plasmid (Promega, Falkenberg, Sweden). The probe was transcribed from supercoiled plasmids, yielding a specific activity of approximately 250 Ci/mmol. A sense

probe from the same plasmid, with a similar specific activity, was used as negative control. Using a previously described procedure,²⁰ except for the omission of conditioning with prehybridization solution, riboprobes were hybridized to sections at 56°C overnight and washed under hybridization-stringent conditions before RNase treatment. After 12 to 48 hours of autoradiography, NTBII photographic emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 in 2% glycerol in H₂O was applied, followed by exposure for 3 to 5 days. The slides were then developed and counterstained for 1 minute with hematoxylin.

Immunohistochemistry

Rat endothelial cells express α -D-galactosyl residues on their surface that can be detected by lectin histochemistry with the lectin *Bandeiraea Simplicifolia* agglutinin (BS-1).²¹ Sections were blocked in 5% normal goat serum in 0.1% bovine serum albumin (BSA) for 1 hour. Lectin (BS-1, biotinylated, Product No. L-3759, Sigma) was applied 1:10 for 1 hour. Solutions were diluted in PBS. For detection, ABComplex/AP (K 391, Dako, Glostrup, Denmark), 1:500, was applied for 30 minutes, followed by development with New Fuchsin (K 698, Dako) and counterstaining with hematoxylin. Sections from purified bovine adrenal capillary cells served as positive controls, and omission of the lectin as negative controls.

To quantify tumor cell proliferation, staining for the Ki 67 nuclear antigen was performed. The monoclonal antibody MIB 1 reacts selectively with the Ki 67 epitope in nuclei of proliferating cells in all phases of the cell cycle except the G₀ phase.²² Sections were blocked in 0.3% hydrogen peroxide for 20 minutes, microwave-treated for 2 × 7 minutes (750W) in citrate buffer, and blocked in 1% BSA for 10 minutes. The primary antibody (MIB 1, monoclonal mouse anti-Ki 67 nuclear antigen, Dianova, Hamburg, Germany) was applied 1:300 for 1 hour at room temperature. The secondary antibody (polyclonal, biotinylated rabbit anti-mouse immunoglobulins, E 354, Dako) was applied 1:200 for 30 minutes. For detection, ABComplex/HRP (K 355, Dako), 1:200, was applied for 30 minutes, followed by development with diaminobenzidine tetrahydrochloride (Sigma) and counterstaining with hematoxylin. Sections from a lymph node served as positive controls, and omission of the primary antibody as negative controls. All antibodies were diluted in 1% BSA in PBS.

Immunohistochemistry for chromaffin differentiation was performed by staining for TH and CgA.²³ Sections were microwave-treated for 2 × 5 minutes (750W) in citrate buffer. Sections for TH were blocked in 0.3% hydrogen peroxide for 30 minutes and in 1% BSA for 15 minutes. The primary antibody (monoclonal mouse anti-TH, No. 1017 381, Boehringer Mannheim, Mannheim, Germany) was applied 1:40 overnight at room temperature. The secondary antibody (polyclonal, biotinylated rabbit anti-mouse immunoglobulins, E 354, Dako) was applied 1:200 for 30 minutes. For detection ABComplex/HRP (K 355, Dako), 1:100, was applied for 30 minutes, followed by development with diaminobenzidine tetrahydrochloride. IHC for CgA was performed by an auto-

mated stainer (Ventana ES, Ventana Medical Systems, Tucson, AZ). The primary antibody (monoclonal mouse anti-human CgA, No. 1199 021, Boehringer Mannheim) was applied 1:2000 for 28 minutes at 42°C. Steps performed by the instrument include blocking with BSA, application of a secondary antibody conjugated to the avidin-biotin peroxidase complex, and visualization with diaminobenzidine tetrahydrochloride as a substrate. All sections were counterstained with hematoxylin. Sections from an adrenal medulla (stained with TH) or human jejunum (stained with CgA) served as positive controls, and omission of the primary antibodies as negative controls. All antibodies were diluted in 1% BSA in PBS.

TUNEL

TUNEL detects typical DNA fragmentation during apoptosis, the energy-dependent process of cell death.²⁴ After deparaffination, sections were digested by proteinase K (20 μ g/ml) for 15 minutes. After four washes in distilled water and blocking in 2.0% hydrogen peroxidase in PBS, the Apotag kit (Oncor, Gaithersburg, MD) was applied according to the manufacturer's instructions. As a positive control, DNase I was added (20 minutes at 37°C) after blocking in hydrogen peroxidase, thus producing DNA breaks in virtually all cells.²⁴ Terminal deoxynucleotidyl transferase replaced with water served as a negative control. The slides were counterstained with hematoxylin.

Scanning Electron Microscopy

For analysis of microvascular casts, tissue was removed from the methacrylate replicas by differential corrosion,¹⁸ dried in air from distilled water, mounted on aluminum stubs, sputter-coated with a 450-Å gold layer, and observed in a Philips 525 scanning electron microscope at an acceleration voltage of 4–10 kV.

Stereological Quantification

Sections from each perfused tumor, stained with either hematoxylin and eosin or BS-1, were used. All sections were coded before quantification. Structures were counted at ×400 with an eyepiece grid (506800, Leica, Singapore) of 10 × 10 squares (0.25 × 0.25 mm). The grid was placed at random at the upper left corner of the section and systematically advanced every 1 to 3 mm (depending on the tumor size) in both directions by use of the goniometer stage. Morphological parameters of 15 to 40 grids were quantified from each tumor.

To adjust for the presence of apoptotic, necrotic, and hemorrhagic areas, the presence or absence of viable tissue in the uppermost square to the far right of the grid was noted (n_{VC} = number of grids with "viable corner") and used in the calculation of vascular parameters. The value of n_{VC} was also used as an unbiased estimator of the fraction of viable tumor tissue. Definitions of vascular parameters are given in Table 1. The procedures have been reviewed by Weibel²⁵ and Gundersen et al.²⁶

Table 1. Definitions of Vascular Parameters

Symbol	Dimension	Equation	Definition
$L_V(\text{ves})$	mm^{-2}	$L_V(\text{ves}) = 2 \times \Sigma Q(\text{ves})/n_{VC} \times A(\text{frame})$	Length of vessels per tumor volume (length density)
$V_V(\text{ves})$	1	$V_V(\text{ves}) = \Sigma P(\text{ves})/n_{VC} \times P(\text{pcg})$	Volume of vessels per tumor volume (volumetric density)
$S_V(\text{ves})$	mm^{-1}	$S_V(\text{ves}) = 2 \times \Sigma l(\text{ves})/n_{VC} \times L(\text{lig})$	Surface area of vessels per tumor volume (surface density)
$\bar{a}(\text{ves})$	mm^2	$\bar{a}(\text{ves}) = V_V(\text{ves})/L_V(\text{ves})$	Mean section area of vessels
$\bar{b}(\text{ves})$	mm	$\bar{b}(\text{ves}) = S_V(\text{ves})/L_V(\text{ves})$	Mean boundary length of vessels
$\bar{d}(\text{ves})$	mm	$\bar{d}(\text{ves}) = 2 \times \sqrt{\bar{a}(\text{ves})/\pi}$	Mean section diameter of vessels

The eyepiece grid was used as an unbiased counting frame, a point-counting grid, and a line-intercept grid.
 $Q(\text{ves})$, number of vessel profiles in one counting frame; n_{VC} , number of grids with viable tissue in the uppermost square to the far right; $A(\text{frame})$, area of one counting frame; $P(\text{ves})$, number of test points hitting vessels (any layer or lumen) in one point-counting grid; $P(\text{pcg})$, number of test points in one point-counting grid; $l(\text{ves})$, number of intersections with vessels in one line intercept grid; $L(\text{lig})$, length of test lines in one line-intercept grid.

The percentages of Ki 67- and TUNEL-positive tumor cell nuclei and TH-positive tumor cells were determined from a minimum of 2000 cells in each specimen. *IGF2* expression was quantified for a minimum of 250 squares (1 square = 1/100 grid) with viable tissue in each specimen by using the same grid but counting only in the upper right quadrant of each grid. The results are expressed as the percentage of squares with more silver grains than the background in relation to all squares.

Statistics

Data were processed in Statistica 4.1 (StatSoft, Tulsa, OK) on a Macintosh PowerBook 540c personal computer. Differences between groups were analyzed with the Mann-Whitney *U* test. Correlations between variables were calculated with Spearman's rank correlation coefficient.

Results

In Vitro Effects of TNP-470

To rule out the possibility that TNP-470 has effect on chromaffin differentiation of the neuroblastoma cells, *in vitro* analysis using the 4-chamber slides was performed. There was no effect on chromaffin differentiation (ie, TH and *IGF2*) of neuroblastoma cells at the concentrations tested (0–1 $\mu\text{g}/\text{ml}$ of TNP-470).

Tumor Angiogenesis

The tumors in animals treated with TNP-470 were 66% smaller than those in controls, but the fraction of viable

tumor cells was also reduced by 33%. Hence, the tumorigenic effect was even better than the effect on the measured tumor volume. Tumor volume doubling time was 5.6 days, compared to 3.3 days in controls. Stereological quantification of vascular parameters was performed on perfused vessels, which appeared as punched-out holes in sections. To check the validity of this procedure, BS-1 staining of endothelial cells was performed. Tumor cells forming vascular channels cannot be excluded, but cells lining blood vessels exhibited BS-1 binding. In clinical cancers, the vast majority of cells lining blood vessels express the endothelial markers factor VIII-related antigen, CD31, and CD34. There was no difference between quantification of perfused vessels and of BS-1 stained vessels (data not shown), indicating that all blood vessels were quantified. The results are presented in Table 2. Note that only the relative values (number of structures per volume, ie, L_V , V_V , and S_V) were significantly reduced, while the absolute values (ie, $\bar{a}$, $\bar{b}$, and $\bar{d}$) were unchanged (Figure 1).

Apoptosis and Proliferation

The proliferative index was similar for control and TNP-470-treated tumors (Table 3). However, the apoptotic index was increased more than threefold in the latter tumors (Table 3). TNP-470-treated tumors displayed a sleeve-like arrangement of neuroblastoma cells surrounding a central vessel, forming perivascular cuffs some 10 to 15 cell layers thick. Proliferating cells were located mainly in the inner and middle layers of the perivascular cuffs, whereas apoptotic cells were confined to the outer layers (Figure 2, A and B). Control tumors showed a similar pattern of cell distribution, al-

Table 2. Quantification of Tumor Angiogenesis in the Control and TNP-470-Treated Tumors (Mean $\pm$ SE)

	Control $n = 14$	TNP-470 $n = 15$	Change	<i>P</i>
$L_V (\text{mm}^{-2})$	44.7 ± 2.7	28.4 ± 2.9	–36%	<0.001
$V_V (10^{-3})$	32.5 ± 3.6	18.0 ± 2.8	–45%	<0.01
$S_V (\text{mm}^{-1})$	3.5 ± 0.2	2.2 ± 0.2	–37%	<0.001
$\bar{a} (10^{-4} \times \text{mm}^2)$	7.5 ± 0.9	7.2 ± 1.3		n.s.
$\bar{b} (\mu\text{m})$	78.3 ± 5.0	82.3 ± 8.1		n.s.
$\bar{d} (\mu\text{m})$	30.2 ± 1.8	28.5 ± 2.7		n.s.

Vascular parameters are defined in Table 1.

Figure 1. Microvascular corrosion cast at scanning electron microscopy. Human neuroblastoma xenotransplanted to nude rat. **A:** control; **B:** treated with TNP-470. **Top:** overview; **bottom:** close-up of the tumor capsule. The tumor microcirculation consists of newly formed sinusoidal tumor vessels, 10–80 μm in diameter, exhibiting extensive anastomoses. Only the number of vessels is affected by treatment with TNP-470, not the angioarchitecture. There are a few true arteriovenous capillaries, 4–10 μm in diameter. Scale bars: overview, 1 mm; close-up, 500 μm .

though the viable tumor tissue was more dense and was less markedly separated into vascular cuffs by masses of apoptotic and necrotic cells and hemorrhages.

Chromaffin Differentiation

In TNP-470-treated tumors, cells in the middle and outer layers of the perivascular cuffs exhibited signs of developmentally specific extra-adrenal chromaffin differentiation; ie, they showed development of cellular processes in combination with induction of expression of *IGF2* and TH (Table 3 and Figure 2, C-E). Cellular processes were

best visualized with the TH IHC staining (Figure 2F). CgA immunoreactivity was present in all cells, so that the increased reactivity in cells in the outer layers of the perivascular cuffs could not be quantified stereologically (Figure 2G). We therefore measured the plasma CgA concentration in rats with and without TNP-470 treatment to evaluate the neuroendocrine tumor activity (Figure 3). The pattern of sudden cellular differentiation at an approximate distance of 100 μm from the central vessel followed by apoptosis in the cuff periphery was most evident in TNP-470-treated tumors (Table 3). It is noteworthy that these differentiated cells in the cuff periphery

Table 3. Quantification of Tumor Dynamics and Differentiation in the Control and TNP-470-Treated Tumors (Mean $\pm$ SE)

	Control	TNP-470	<i>n</i>	Change	<i>P</i>
Tumor volume (ml)	6.7 $\pm$ 0.5	2.3 $\pm$ 0.2	18/15	–66%	<0.001
Viable tissue (%)	77.2 $\pm$ 3.2	51.5 $\pm$ 3.0	14/15	–33%	<0.001
Proliferative cells (%)	48.7 $\pm$ 0.5	49.1 $\pm$ 0.7	7/7		n.s.
Apoptotic cells (%)	2.1 $\pm$ 0.2	6.7 $\pm$ 0.5	7/7	+219%	<0.01
<i>IGF2</i> expression (%)	24.0 $\pm$ 1.8	45.2 $\pm$ 1.9	6/6	+88%	<0.01
TH-positive cells (%)	11.5 $\pm$ 1.2	22.5 $\pm$ 2.8	7/6	+96%	<0.01

IGF2, insulin-like growth factor II gene; TH, tyrosine hydroxylase.

Figure 2. Markers of proliferation, apoptosis, and chromaffin differentiation in neuroblastoma tumors treated with TNP-470, perivascular cuffs. **A:** Ki 67; proliferative cells in the inner and middle layers. **B:** TUNEL; apoptotic cells mainly located in the outer layers. **C:** Insulin-like growth factor II gene (*IGF2*) expression, dark field view. Note the distinct ring-like expression peripheral to the central vessel. **D:** *IGF2* expression, light field view. **E:** TH; positive cells located in the middle and outer layers, essentially the same distribution as *IGF2*. **F:** TH; Cellular processes (arrows) in close-up. **G:** Chromogranin A. **H:** Hematoxylin and eosin. Scale bars: F, 20 μ m; all others, 100 μ m.

would have been classified as poorly differentiated if conventional criteria based solely on hematoxylin and eosin stainings had been used (Figure 2H). Control tumors exhibited a similar pattern of chromaffin differentiation but, again, this was less pronounced because of the dense tumor tissue.

Correlation between Tumor Angiogenesis and Chromaffin Differentiation and Apoptosis

Regression analysis with Spearman's rank correlation coefficient revealed significant inverse correlations between *IGF2* expression and all vascular parameters (L_V , $r = -0.79$, $P < 0.01$; V_V , $r = -0.85$, $P < 0.001$; S_V , $r = -0.77$, $P < 0.01$). Inverse correlations were also found between

one of the vascular parameters and both TH-positive cells (L_V , $r = -0.61$, $P < 0.05$) and apoptotic cells (V_V , $r = -0.53$, $P < 0.05$). With the same analysis, the other two vascular parameters showed no significant correlations.

Discussion

The angiogenesis inhibitor TNP-470 has been shown to reduce tumor growth and vascularization in syngeneic and xenogeneic animal experimental models.^{10,27} On the tumor cell level, the proliferation index is generally unaffected but the apoptotic index is increased.^{11,28} These observations were confirmed in our model, using stereo-

Figure 3. Plasma concentration of chromogranin A (CgA) versus tumor volume. CgA levels were directly proportional to tumor volume and intact CgA was liberated to the rat circulation from viable tumor cells, as we have reported earlier.⁴² Treatment with TNP-470 (in a separate correlation study) did not affect this correlation, despite a 33% reduction of viable neuroblastoma cells. Our interpretation of this phenomenon is that TNP-470 causes increased CgA secretion as a sign of chromaffin differentiation. CgA was measured with a specific competitive radioimmunoassay.⁴³ The heparin plasma was kept frozen until measurements. ●, Treatment with 10 mg/kg body weight of TNP-470 s.c. every other day for 12 to 24 days ($n = 7$); ○, no treatment ($n = 20$, control group).

logical quantification of angiogenesis and cellular dynamics. A threefold increase in the apoptotic index can explain a reduced net growth of the tumor if the proliferative index is constant. Moreover, biological analysis of neuroblastoma cells in TNP-470-treated animals revealed tumor cell differentiation. This is the first report on such an effect of an angiogenesis inhibitor.

In vitro, TNP-470 restrains endothelial cell proliferation at concentrations much lower (10 pg/ml) than those inhibiting neuroblastoma cell proliferation (10 μ g/ml).^{8,16} *In vivo*, the tumorstatic effect of TNP-470 is mediated by its angiostatic activity rather than by a direct effect on tumor cell proliferation.¹⁰ The plasma concentrations of TNP-470 measured in nude rats by reversed-phase high-pressure liquid chromatography after s.c. injections of similar doses are <1 μ g/ml (Katsuichi Sudo, Takeda, data on file). TNP-470 had no effect on differentiation. We found that TNP-470 therapy reduced stereological vascular parameters by 36–45%. We conclude that in our neuroblastoma model the effect of TNP-470 is angiostatic and not cytotoxic (this report and Wassberg et al¹⁶).

However, TNP-470 potentiates the tumorstatic effects of cytotoxic drugs and external radiation in animal experimental models.^{6,7} This synergistic effect has been claimed to be due to a compensatory dilatation of established, nongrowing tumor vessels by TNP-470 treatment.²⁹ This dilatation would lead to local increases in perfusion and oxygenation in the tumor, thereby reducing anaerobic metabolism and increasing radiosensitivity. This hypothesis could not be validated in our experiments

Figure 4. Agonal differentiation and apoptosis. Based on the cellular dynamics and differentiation observed, we suggest that TNP-470 induces metabolic stress (eg, hypoxia, reduction of endothelium-derived growth factors), leading to agonal chromaffin differentiation and apoptosis of neuroblastoma cells. Abbreviations: Ki 67, proliferating cells; TUNEL, apoptotic cells; IGF2, insulin-like growth factor II gene; TH, tyrosine hydroxylase; CgA, chromogranin A.

because the mean tumor vessel diameter was similar in treated tumors and controls.

In our model, TNP-470-treated tumor cells formed cuffs around residual vessels. The inner cellular layers exhibited the normal SH-SY5Y phenotype,¹⁶ whereas the middle and outer layers showed induction of expression of IGF2 and TH. The outer layers displayed increased expression of CgA and various stages of apoptosis. This pattern is similar to that reported in clinical neuroblastomas.¹⁵ Recent data have shown that clinical neuroblastomas more specifically differentiate into the type I small intensely fluorescent cell lineage, an extra-adrenal chromaffin cell type with combined neuronal and endocrine features which is most abundant during development and early childhood (F Hedborg, G Franklin, J Norrman, L Grimelius, E Wassberg, F Schilling, D Harms, B Hero, F Berthold, B Sandstedt, unpublished manuscript). In the present study, evidence for this combined neuronal and endocrine phenotype was provided by the formation of cellular processes with endocrine features. It is concluded that inhibition of angiogenesis induces chromaffin differentiation and apoptosis in neuroblastoma.

The mechanism by which TNP-470 induces differentiation and apoptosis is not clear. We hypothesize that inhibition of angiogenesis induces metabolic stress. This is based on the morphological findings and the inverse correlations of angiogenesis with differentiation and apoptosis. We define metabolic stress here as the aggregate effect of factors such as hypoxia, hypercapnia, reduced pH, and hypoglycemia, as well as reduction of endothelium-derived growth factors acting in a paracrine fashion on the tumor cells. The metabolic stress makes neuroblastoma cells respond by differentiation before their death by apoptosis. We propose the term agonal differentiation for this process (Figure 4). The differentiation observed is likely to be a secondary effect of metabolic stress induced by inhibition of angiogenesis.

Hypoxia may serve as a mediator between angiostatic therapy and differentiation with apoptosis. This hypothesis is supported by the finding that anoxia can induce

chromaffin differentiation in SH-SY5Y cells *in vitro* (F Hedborg, G Franklin, J Norrman, L Grimelius, E Wassberg, F Schilling, D Harms, B Hero, F Berthold, B Sandstedt, unpublished manuscript). Hypoxia itself also induces apoptosis.³¹ At the same time, hypoxia can induce expression of the angiogenic peptide vascular endothelial growth factor (VEGF).^{31,32} Recent data also show that hypoxia may be a stimulus for induction of expression of *IGF2*.³³ In our model, an up-regulation of VEGF mRNA was observed in the middle and outer layers of the perivascular cuffs in the TNP-470-treated tumors (data not shown). However, this signal for angiogenesis by VEGF was abolished by TNP-470 because all vascular parameters were reduced. An inverse correlation between apoptosis and angiogenesis has been demonstrated in human gastric carcinoma.³⁴ Such a correlation was confirmed in our experimental model, as was an inverse correlation between differentiation and angiogenesis. Taking this together with the morphological pattern, an oxygen gradient from the central vessel could be a mediator of the differentiation and apoptosis observed.

Besides hypoxia, another mediator of differentiation and apoptosis could be the reduction of endothelial cell-derived trophic factors, at least 20 of which are defined and known to be expressed by tumor endothelium.³⁵ It is possible that TNP-470, by reducing the endothelial compartment (in our experiments by 36–45%), also reduces endothelial-derived growth factors and other cytokines. Growth factor and/or cytokine withdrawal may be a mechanism for tumor cell gene activation leading to differentiation and apoptosis. Removal of growth factors or induction of differentiation have been shown to induce apoptosis *in vitro*.³⁶ We would like to emphasize that agonal differentiation precedes and may be necessary for apoptosis. Whether reduction of trophic factors mediates differentiation and apoptosis in neuroblastoma is not yet known, but the question is clearly amenable to future investigations.

Chromaffin differentiation and apoptosis are of particular interest, because neuroblastoma is one of the few human cancers that may regress spontaneously (eg, the stage IV-S group of tumors and a significant proportion of the tumors identified via infant mass screening).³⁷ The morphology of spontaneous regression in neuroblastoma has not been satisfactorily investigated but, histologically, calcifications,³⁸ apoptotic areas, and chromaffin differentiation have been regarded as good prognostic signs.^{39,40} Neuroblastoma cells, such as the SH-SY5Y cell line, can be induced to differentiate *in vitro* by, for example, phorbol esters and retinoic acid derivatives.⁴¹ Differentiation in cultured cells as well as in clinical neuroblastomas involves a reduced growth rate, expression of *IGF2*, TH, and CgA, and development of cellular processes with endocrine features and neuropil.^{15,23,41} (F Hedborg, G Franklin, J Norrman, L Grimelius, E Wassberg, F Schilling, D Harms, B Hero, F Berthold, B Sandstedt, unpublished manuscript). This indicates that TNP-470 in combination with retinoic acid may be beneficial. Neuroblastoma differentiation and apoptosis are therefore important for both prognostication and new treatment strategies.

Induction of differentiation is a newly discovered effect of an angiogenesis inhibitor. This agonal differentiation may be specific for neuroblastoma, an embryonic cancer capable of spontaneous differentiation. But is agonal differentiation before apoptosis a general pattern of angiostatic therapy? Such a phenomenon could have remained undetected due to lack of specific differentiation markers. This intriguing question demands further investigation because it may reveal new targets for therapy.

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