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Angiostatic Treatment of Neuroblastoma

Minireview based on a doctoral thesis

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ABSTRACT

Neuroblastoma is a malignant solid tumor of childhood with a poor prognosis. The growth of solid tumors has been shown to be dependent on new blood vessel formation, i.e. angiogenesis. Several steps in the metastatic process have also been found to be angiogenesis-dependent. Neuroblastomas grow quickly, are highly vascularized, and metastasize early, and hence inhibition of angiogenesis — angiostatic therapy — may be indicated in this disease. In order to investigate the effects of angiostatic agents in this disease, a new animal experimental model for human neuroblastoma was developed. Three angiostatic agents were tested in the model: TNP-470, the synthetic analogue of fumagillin, given subcutaneously, and the endogenous steroid 2-methoxyestradiol and its derivative 2-propynylestradiol, given orally. TNP-470 administration resulted in a significant reduction of the tumor growth rate and microvascular counts, and of the fraction of viable tumor cells, compared to controls. The fraction of apoptotic tumor cells increased threefold, while that of proliferative cells remained unaltered. This can explain the reduced net growth. Treatment with the angiostatic and chemotherapeutic steroids 2-methoxyestradiol and 2-propynylestradiol yielded similar results. However, the mechanism of action of these steroids was bimodal; the effect occurring both through inhibition of tumor angiogenesis and through induction of tumor cell apoptosis. It was shown for the first time that inhibition of angiogenesis regardless of agent induces striking chromaffin differentiation, observed as increased expression of insulin-like growth factor II gene, tyrosine hydroxylase, and chromogranin A, and increased formation of cellular processes. It is suggested that inhibition of angiogenesis induces metabolic stress, resulting in chromaffin differentiation and apoptosis. Such agonal differentiation may be the link between angiostatic therapy and tumor cell apoptosis. Angiostatic agents administered as single therapy have an objective tumoristatic effect in our neuroblastoma model. Angiostatic treatment of neuroblastoma is a new and promising treatment modality that merits clinical investigation.

Key words: Angiogenesis — Angiostatic treatment — Apoptosis — Differentiation — Neuroblastoma.

Abbreviations

BS-1	Bandeiraea Simplicifolia agglutinin
bFGF	basic fibroblast growth factor
CgA	chromogranin A
HVA	homovanillic acid
IGF2	insulin-like growth factor II gene
$2ME_2$	2-methoxyestradiol
NSE	neuron-specific enolase
$2PE_2$	2-propynylestradiol
TH	tyrosine hydroxylase
VEGF	vascular endothelial growth factor
VMA	vanillyl mandelic acid

INTRODUCTION

Background

New blood vessel formation — angiogenesis — occurs physiologically during placentation, embryonic and fetal growth, corpus luteum formation, and rebuilding of the endometrium after menstruation. Angiogenesis is also the hallmark of wound healing. In a healthy adult, however, angiogenesis is a rare event and vascular endothelial cells generally exhibit a slow turnover. Almost three decades ago clinical observations and experimental studies led to the suggestion that tumor growth, just like the growth of any other tissue, is dependent on angiogenesis (1). Hence, a tumor does not rely on invasion of pre-existing vessels for expansion of the tumor cell population, but triggers the surrounding tissue to form new blood vessels to converge upon the tumor. There is now substantial evidence that tumor growth and metastasis are angiogenesis-dependent (for reviews, see refs. 2-10).

Angiogenesis is a tightly controlled and complex process involving several factors on the molecular and cellular levels, with both stimulating and inhibiting steps. As yet, no simple wiring diagram for the process can be made. Once initiated, however, angiogenesis is characterized by a rapid and efficient cascade of events involving factors such as endothelial cell proliferation and migration, dissolution of vascular basal membranes, increased vascular permeability, and degradation of the extracellular matrix. The process by which malignant cells acquire their angiogenic phenotype and stimulate angiogenesis is now being unravelled (4, 7). Tumor angiogenesis is controlled both by genetic and by physiological events (11). As tumor cells progress toward tumorigenicity, they acquire the ability to induce angiogenesis as a result of the activation of oncogenes — which often stimulate the secretion of angiogenesis stimulators — and inactivation of tumor suppressor genes, which frequently leads to a decrease in the production of inhibitors of angiogenesis. Also, the tumor progression itself can lead to an increase in circulating

levels of activators, e.g. induction of VEGF by hypoxia (12). This angiogenesis cascade, although complex, is also well conserved from the evolutionary aspect.

Neuroblastoma is the most common extracranial malignant solid tumor of childhood. It arises from the sympathetic nervous system (13). The prognosis in infants and children with neuroblastoma is dependent on the age of the child and the clinical stage of the tumor at diagnosis, younger patients with small tumors having the best prognosis (14). In spite of aggressive therapeutic protocols involving high-dose chemotherapy and total body irradiation, followed by bone marrow transplantation, the overall survival of patients with advanced disease (i.e. stages III and IV) remains poor (15). Clinically, there is a need for more effective treatment modalities. The invasive, metastatic, and hypervascular nature of these tumors may be one of the key obstacles to the cure of this disease (Fig. 1). Hence, it is likely that neuroblastomas elaborate angiogenic peptides and thus would respond to angiostatic treatment.

Angiostatic drugs in neuroblastoma

Inhibitors of angiogenesis are now being identified at an increasingly rapid rate (16, 17). Hitherto, at least 13 angiostatic agents of different structure and with different modes of action have entered clinical trials (Tables I and II). Angiogenesis inhibitors currently undergoing phase I, II and III clinical trials represent the first generation of angiostatic agents. This means that not only will it take time until the efficacy — and side effects — of these inhibitors are known, but it will also take even longer until the next generation of angiostatic agents, e.g. angiostatin (18) and endostatin (19), have completed clinical trials. Angiostatin and endostatin have been shown to induce tumor regression and tumor dormancy without drug resistance in several experimental models (20, 21). In clinical practice, however, angiostatic agents will perhaps be administered in combination with other therapies, since effects synergistic with those both of cytotoxic agents and of radiotherapy have been observed experimentally (22, 23). These observations suggest that therapy targeting both endothelial cells and tumor cells is more effective than therapy directed against tumor cells alone. Also, two or more angiogenesis inhibitors may be combined to obtain a tumor response. Again, such potential treatment will require several clinical investigations to be optimized. In conclusion, inhibition of angiogenesis is a new treatment modality in cancer, and merits a thorough evaluation.

An anticipated side effect of angiogenesis inhibition in pediatric patients is impairment of organ development and growth. Hopefully, angiostatic agents which specifically inhibit tumor-induced angiogenesis and not physiological angiogenesis will be developed. There are promising candidate target molecules that may be expressed only in tumor-induced blood vessels (24-26). In this perspective, however, most treatment modalities today impair growth, and certain chemotherapy

Fig. 1. Human neuroblastoma xenotransplanted to nude rat. Microvascular corrosion cast at scanning electron microscopy. Overview of the tumor capsule. None of these vessels existed before the tumor, all are newly formed and a visual impression of extensive angiogenesis is obtained. The casts are made by resin infusion through the thoracic aorta. The tumor microcirculation consists of sinusoidal tumor vessels, 10-80 μm in diameter, exhibiting extensive anastomoses. There are comparatively few true arteriovenous capillaries, 4-10 μm in diameter. Bar=500 μm.

Table I. Selected angiogenesis inhibitors and approaches under study.

Agent	Company or Institution	Clinical trial	Refs.
Angiostatin	EntreMed Bristol-Myers Squibb	—	
Batimastat (BB94)	British Biotechnology	Phase II	73
Carboxyamidotriazole	National Cancer Institute	Phase II	74
CM101	Carbomed and Zeneca	Phase I	75
Endostatin	EntreMed Bristol-Myers Squibb	—	
Interferon α	Various centers	Phase III	76
Interleukin-12	Hoffman – La Roche	Phase II	77
Linomide	Pharmacia & Upjohn	Phase I	78
Marimastat	British Biotechnology	Phase III	73
2-Methoxyestradiol	EntreMed	—	
Platelet factor 4	RepliGen	Phase II	79
Soluble FLT-1 VEGF receptor	Merck Research Laboratories	—	
SU5416	Sugen	Phase I	80
Suramin	Various centers	Phase II	81
Tecogalan (DS-4152)	Daiichi Pharmaceuticals	Phase I	82
Thalidomide	EntreMed and Celgene	Phase II	83
TNP-470	Takeda	Phase III	84
Vitaxin	IXSYS	—	

Except for refs. (75, 80), and (81), complete citations are given in refs. (2-4, 10), and (85). For the latest update, visit http://cancernet.nci.nih.gov/ (for clinical trials in USA) or http://telescan.nki.nl/ (for clinical trials in Europe).

regimens even have serious long-term side effects such as cardiotoxicity and induction of secondary malignancies (27). A specific property of angiostatic therapy is that the agent has to be administered for a long period of time, presumably years, since its effect is cytostatic and not cytotoxic (28). There is a risk that dormant micrometastases (18, 29) may start to grow if angiogenesis inhibition is withdrawn too early. Other possible side effects of angiostatic treatment could be impairment of wound healing and interference with the female reproductive system (30). In short, angiostatic agents are likely to exhibit less systemic toxicity than chemotherapy. Their effects on growth, wound healing and reproduction will be dependent on their specificity for tumor angiogenesis.

Table II. Some angiogenesis inhibitors classified by their proposed mechanism of action.

Mechanisms of action	Agents
Activate the immune system	CM101
Block the production or export of angiogenesis stimulators (bFGF, VEGF)	Interferon α
Stimulate the formation of angiogenesis inhibitors	Interleukin-12
Block the binding or prevent oligomerization of receptors of angiogenesis stimulators	Platelet factor 4 Suramin SU5416 Thrombospondin VEGF receptor antagonists
Inhibit degradation of the endothelial cell basement membrane	Angiostatic steroids plus heparin Cartlage-derived inhibitor Metalloproteinase inhibitors (Batimastat, Marimastat) 2-methoxyestradiol
Inhibit endothelial cell proliferation	Angiostatin Carboxyamidotriazole Endostatin Inhibitors of $\alpha_v\beta_3$ integrins (Vitaxin) Interferon α 2-methoxyestradiol Tecogalan Thrombospondin TNP-470 16-kD prolactin fragment
Induce apoptosis of endothelial cells	2-methoxyestradiol
Inhibit endothelial cell migration	Angiostatic steroids plus heparin Carboxyamidotriazole Interferon α 2-methoxyestradiol Linomide Platelet factor 4 Suramin TNP-470

Constructed from refs. (2, 3, 10, 16), and (85). References for each compound can be found there. This list is not all inclusive and, of course, does not include many potent angiostatic agents whose mechanism is not yet known. Many agents target more than one compound of the angiogenic process.

Potential advantages of angiostatic therapy over chemotherapy are:

I. Angiostatic therapy generally has low toxicity. It is directed mainly at proliferating capillary endothelial cells and does not cause bone marrow suppression, drug-induced enteritis or hair loss in patients, as shown for platelet factor 4 and TNP-470, for example (31). Angiostatin and endostatin have not shown any toxicity in animal models (18, 19).

II. The development of drug resistance is less likely to occur, and has not yet been observed experimentally (2, 4, 8). This is because the target cells are normal, untransformed endothelial cells, in contrast to tumor cells, which are genetically unstable. Drug resistance does not appear to be a problem even with long-term therapy, either in animals (21) or in humans (10).

III. Solid tumors have barriers to drug delivery (e.g. high interstitial pressure), and many tumors resist full penetration by anticancer drugs (32). The endothelial cells are by definition exposed to the circulation — in contrast to most tumor cells — and hence drug delivery is likely to be successful. Angiostatic agents do not have to cross the blood-brain barrier, for example.

IV. Once new capillary loops converge toward a small in situ carcinoma or a microscopic metastasis, the tumor cells become bathed in additional survival factors and growth factors, not only from the circulating blood (perfusion effect) but also from vascular endothelial cells themselves (paracrine effects) (10). The paracrine effect of tumor vascularization results from endothelial-derived growth factors and cytokines which stimulate growth and migration of tumor cells; thus inhibition of angiogenesis would reduce this growth stimulus.

V. The metabolism of 10^4 tumor cells depends on a single capillary loop (33). Regardless of the error inherent in such calculations, they reveal an amplification factor that may operate during angiostatic therapy; that is, even a limited reduction of the number of tumor vessels will affect a large number of tumor cells (10). This snowball effect has nicely been demonstrated by tumor infarction in mice by antibody-directed targeting of tissue factor to tumor vasculature (24, 26).

In conclusion, angiostatic therapy has at least five theoretical advantages over chemotherapy, advantages which still need to be further evaluated from a clinical perspective.

Table III. Angiogenic peptides

Peptide	molecular weight in kD
Acidic fibroblast growth factor	16.4
Angiogenin	14.1
Angiopoietin-1	70
Basic fibroblast growth factor	18
Granulocyte colony-stimulating factor	17
Hepatocyte growth factor	92
Interleukin-8	40
Leptin	16
Neuropeptide Y	4.6
Placental growth factor	25
Platelet-derived endothelial cell growth factor	45
Proliferin	35
Transforming growth factor α	5.5
Transforming growth factor β	25
Tumor necrosis factor α	17
Vascular endothelial growth factor	45

Except for angiopoietin-1 (86), leptin (87, 88) and neuropeptide Y (72), complete citations are given in refs. (2) and (4).

Other potential clinical applications

In addition to inhibition of angiogenesis, two other clinical applications of angiogenesis research to oncology have emerged namely, monitoring of disease activity by analysis of circulating angiogenic peptides, and prediction of an outcome by performing tumor microvascular counts. To date, at least 16 angiogenic peptides (Table III) and most of their endothelial cell receptors have been sequenced and cloned. Apart from these angiogenic peptides, there are also agents of low molecular weight with angiogenic activity. Of the peptides, bFGF and VEGF are the most well studied and are found in a variety of different types of human tumors (34, 35).

The concentration of the angiogenic peptide bFGF in the urine has been found to be frequently elevated in patients with a spectrum of different tumors (36). The urine levels of bFGF correlated to tumor stage and prognosis in children with Wilms´ tumor (37). Urine bFGF was also elevated at the time of diagnosis and decreased with treatment in childhood acute lymphoblastic leukemia (38). The latter phenomenon suggests that leukemias induce angiogenesis in the bone marrow and should be regarded as a solid tumor that can progress for a long time before leukemic cells

become shed to the peripheral blood. Leukemia could therefore also be a candidate for treatment with angiostatic drugs. As yet there are no reports on expression of angiogenic peptides in body fluids in neuroblastoma. However, we are presently investigating the question of whether serum levels of VEGF or bFGF correlate to tumor stage and prognosis in neuroblastoma.

Tumor angiogenesis can be quantified by counting the number of microvessels per surface area in paraffin sections from the resected primary tumor after immunostaining with a suitable endothelial cell marker. Roughly two hundred such investigations have been published, and in most of them a significant correlation was found between a high microvascular count and metastatic disease with a poor prognosis (see refs. 5 and 39 for a review). In the only published report on neuroblastoma (40), the microvascular count correlated with metastatic disease, amplification of the oncogene *MYCN*, and a poor outcome. Since tumor growth is dependent on angiogenesis, it is reasonable to assume that aggressive tumors will exhibit higher microvascular counts than more slowly growing, late-metastasizing tumors.

EXPERIMENTAL DESIGN AND RESULTS

Animal experimental model

To investigate the effects of angiostatic treatment in neuroblastoma, we developed a new animal experimental model (41). In planning the animal model, the following criteria were established: 1) The cell line to be used for xenotransplantation should be human, stable in culture and well charcterized. 2) The cell line should form exponentially growing tumors after subcutaneous injection, since a rapidly growing tumor is likely to be angiogenesis-dependent. 3) The model should yield reproducible results, and should not metastasize. The lack of metastasis has the advantage that it permits simple measurements of the total tumor burden, without applying invasive or imaging techniques.

Cells from the poorly differentiated, adrenergic human neuroblastoma cell line SH-SY5Y (42, 43) were used as tumor xenografts in both nude rats (WAG rnu/rnu) (41, 44, 45) and nude mice (NMRI nu/nu) (46). A suspension of SH-SY5Y cells has been reported to induce exponentially growing solid tumors when injected subcutaneously in nude rats without the tumor cells losing their neuronal phenotype (47). The cell line is stable in culture and well characterized both morphologically and functionally. It lacks amplification of the oncogene *MYCN* (48) as most neuroblastomas do. The neuroblastomal nature of the cultured cells and of the tumors was

determined by immunohistochemistry for specific neuronal markers, i.e. NSE and chromogranin A and B, and transmission electron microscopy (41).

Nude rats were chosen initially because their size is suitable for perfusion fixation and vascular casting, frequent blood sampling, urine sampling, and surgery (41, 44, 45)). Nude rats are athymic and T-lymphocyte deficient. They do not develop cell-mediated immunity during their first three months of life, which means that they cannot reject xenotransplants (49, 50). When we had only a limited amount of expensive agents, we switched to nude mice (46), since their body weight is one tenth of that of the nude rat. The nude mouse is similar to the nude rat regarding its T-lymphocyte deficiency and has been widely used for xenotransplantation of human cancers (51, 52). We chose the nude mouse strain NMRI nu/nu since we found it had a shorter time to tumor take and less variation in tumor growth than the other strains tested (i.e. C57BL/6J, BALB/cABom-nu, and FOX CHASE SCID).

At transplantation, 20 to 30 million tumor cells in a volume of 0.2 to 0.3 ml were injected subcutaneously in the hindleg of the rat or mouse. To ensure tumor take and to minimize variation in tumor take and size, the number of viable tumor cells at transplantation was determined by Trypan Blue exclusion in hemocytometer counts. The concentration of cells at transplantation was 100×10^6 cells/ml and at this concentration the cells retained a high viability for four hours, then rapidly declined. Other cell lines may be more resistant to storage and transport, but in practice, the harvesting of cells and injection of animals must be meticulously planned and accomplished as possible. With these precautions, the tumor take was high, 80-100% in both rats and mice (41, 44-46).

Injection of 40 million cells did not result in a higher take rate (41). A larger volume than 0.4 ml increased the risk of leakage from the injection site and formation of multiple tumors (41). Technically it was found important to tent the skin before injecting, to ensure that the cells were deposited subcutaneously and not intramuscularly or intradermally. This procedure is equally important when injecting drugs subcutaneously. There were no differences in tumor take or growth between animals injected in one hind leg and those injected in both (41). Also, serum samples from 17 tumor-bearing animals were tested on bovine capillary endothelial cells in vitro. The sera had no effect on proliferation of the endothelial cells (Michael O'Reilly, personal communication) (41). This implies that the neuroblastoma tumors do not produce or induce angiogenic stimulators or angiogenic inhibitors in concentrations that will affect a distant tumor in the same animal.

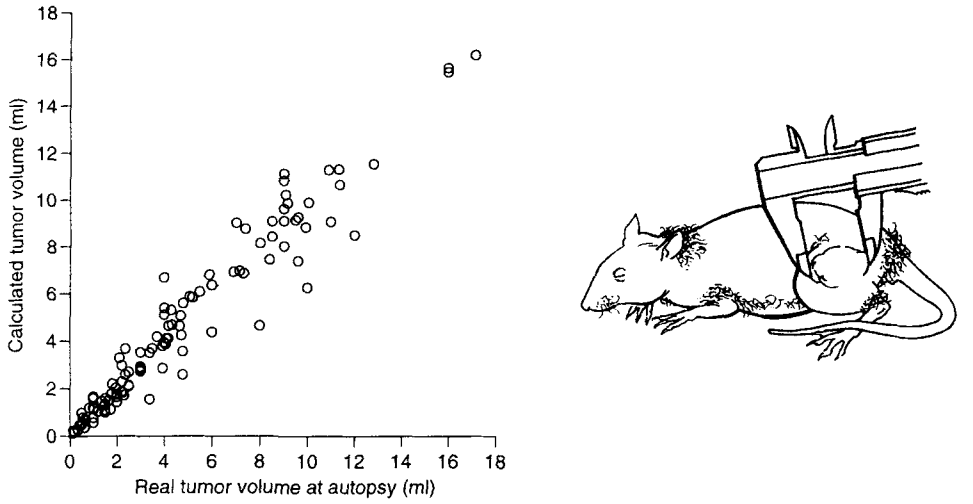


Fig. 2. Calculated tumor volume versus real tumor volume at autopsy in 112 tumors. After perfusion fixation the tumors were dissected out, and their volume recorded, as described by Elias and Hyde (50). Tumors used in the calculation for the constant were those where the dissection was optimal, i.e. the tumor capsule was intact after dissection. Tumor volume was calculated by length x width^2 x 0.44, and the measurements were made with a caliper under halothane anesthesia.

Tumor volume was calculated as length x width^2 x 0.44. The tumor length was measured along the long axis of the tumor, and the width perpendicular to the long axis. The measurements were made with a caliper every other day under halothane anesthesia. We were unable to make accurate measurements without anesthesia. Several algorithms have been proposed for calculating volumes of subcutaneously grafted tumors (53). The constant 0.44 was deduced from our data comprising 112 tumors (Fig. 2), and may be specific for our xenotransplant model only. The tumor height was also measured, but in our hands was not as reproducible and reliable as the tumor length and width. The true tumor volume was recorded at autopsy by the immersion method (54): A container with water was placed on a balance and the weight was recorded. The tumor was then immersed in the water by a thin thread so that it was fully covered and did not touch the bottom of the container. The new weight in grams, minus the weight of the container and water, equals the volume of the tumor in cubic centimeters.

No distant metastases were found in our model (41). At autopsy, samples were also taken for immunohistological detection of metastatic disease in lymph nodes, liver, lungs, and bone marrow showing no microscopic evidence of neuroblastoma growth (41). Also, in six rats no metastatic growth was observed 60 days after removal of the primary tumor. To our surprise, intravenous injection of 60 million cells in five animals did not result in disseminated metastatic growth after a 50-day observation period.

Perfusion fixation

The microcirculation is designed for exchange of nutrients, oxygen, and catabolites between the blood and the cells. It is essentially a network of tubes distributed in three dimensions throughout the tissues. In other words, the best way to fix the tissue would be to use the vascular system for transportation of the fixative. Such perfusion fixation is a comparatively simple method, and since most cells lie close to a vessel (within 200 μm), almost instantaneous fixation is achieved. This is advantageous, since delayed fixation can damage epitopes. At immersion fixation the fixative has to penetrate the tissue through diffusion, which is slower and less efficient. We were not only interested in achieving optimally fixed tissues, however, but also in quantifying tumor vessels. We therefore monitored the intraarterial pressure through a cannula inserted in the femoral artery during perfusion fixation. The pressure was kept between 100 and 140 mm Hg, thereby ensuring reproducibility and avoiding overdistension of vessels as well as incomplete perfusion.

Stereological quantification

There are different ways of quantifying tumor angiogenesis. Thus the methodology varies between studies, which makes it difficult to compare the vascular densities reported. Most investigators quantify structures stained with an endothelial marker. In human cancers, the vast majority of cells lining blood vessels express the endothelial markers factor VIII related antigen (von Willebrand factor), CD31 (PECAM; platelet-endothelial cell adhesion molecule), and CD34 (39). In animal models, however, the newly formed tumor blood vessels derive from the host animal even when the engrafted tumor cells are human. Pilot experiments showed that we could not use antibodies raised against human endothelial epitopes in our model. This meant that other methods for highlighting blood vessels in sections had to be considered. Rat and mouse endothelial cells express, on their surface α-D-galactosyl residues that can be detected by lectin histochemistry with the lectin BS-1 (55). Another approach for visualizing tumor blood vessels in an experimental setting is vascular perfusion as described above. Vascular perfusion makes blood vessels appear as punched-out holes in sections. To check the validity of this approach, BS-1 staining of endothelial cells was also performed in 20 tumors. There was no difference in quantification between perfused vessels and BS-1 stained vessels, indicating that all blood vessels were quantified in practice (Fig. 3) (45). Also, the methods confirmed the results of each other, in that the cells bordering holes in sections showed staining for BS-1, and BS-1-stained structures were perfused (45). In our studies stereological quantification of vascular parameters was performed on perfused vessels throughout.

The definition of vascular density varies in different studies from the number of vessels, over stained endothelial cell area, to total vessel area — including the lumen — per section area. Also,

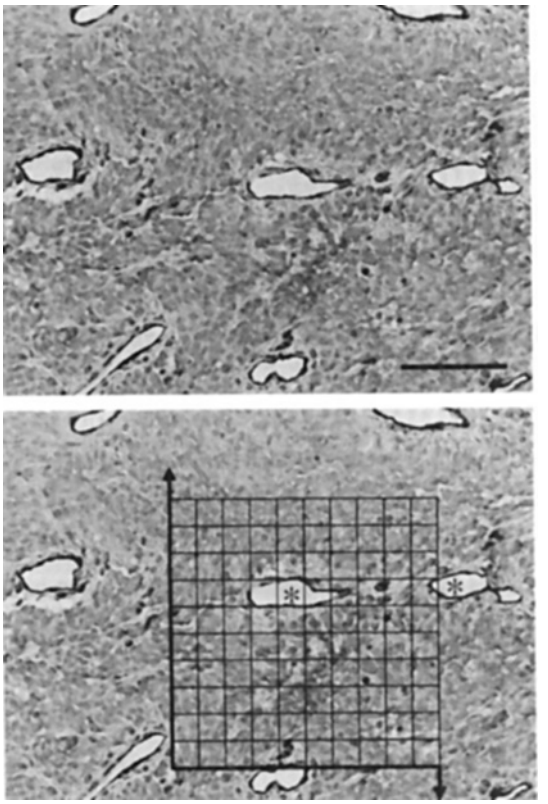


Fig. 3. *Above.* A restricted field of vision of a tumor section with perfused and BS-1 stained vessels. *Below.* The same field with an unbiased counting frame. In addition to vessels completely inside the frame one counts all vessels with anything inside the frame, vessels marked with an asterisk, provided they do not in any way touch or intersect the full drawn exclusion edges or their extensions. Light microscopy. Bar=100 μm.

the area subjected to quantification varies from the entire tumor area, over the tumor hot spot, to random. Moreover, counting of vessels is dependent on the endothelial marker used, the magnification used, and the experience of the investigator, and there is also inter- and intraobserver variability (39). In order to reduce this variability, we tried to reduce observer bias and standardize the counting procedure as much as possible. In addition, to obtain further information on the length of the vessels and their volumetric and surface density, as well as on the vessel area, vessel boundary length, and vessel diameter, we applied an unbiased sterological method for quantification of vascular parameters (45, 46):

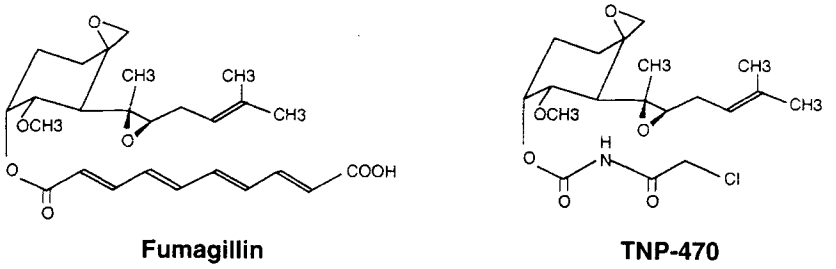
Sections from each perfused tumor were coded before quantification. Structures were counted at × 400, a magnification at which individual capillaries can be discriminated. The microscope was equipped with an eyepiece grid of 10 × 10 squares (250 × 250 μm) (Fig. 3). The grid was placed at random at the upper left-hand corner of each tumor section and systematically advanced every 1 to 3 mm (depending on the tumor size) in both directions by use of the goniometer stage. Thus, areas for quantification were sampled independently of the observer and were evenly distributed

over the entire tumor cross-section. Morphological parameters from 15 to 40 grids were quantified in each tumor. To adjust for the presence of hemorrhages and cellular debris, the presencé or absence of viable tissue in the uppermost square to the far right of each grid was noted and used in the calculation of vascular parameters. This value was also used as an unbiased estimator of the fraction of viable tumor tissue. The procedures used were reviewed by Weibel (56) and Gundersen et al. (57). We initially considered using computer assisted image analysis, but were concerned about the implementation of the programs and their ability to discriminate vascular structures from artifacts. We found that the applied methodology was simple, reproducible, fast and efficient. It can also easily be reproduced by other investigators.

Chromaffin differentiation

Differentiation and apoptosis (programmed cell death) of neuroblastoma cells are of particular interest, since neuroblastoma is one of the few human cancers that may regress spontaneously, i.e. the stage IV-S group of tumors and a significant proportion of the tumors identified via infant mass screening (58). The morphology of spontaneous regression in neuroblastoma has not been satisfactorily investigated, but histologically calcifications (59), apoptotic areas, and differentiation have been regarded as good prognostic signs (60, 61). 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 (48). Differentiation in cultured cells as well as in clinical neuroblastomas involves a reduced growth rate, expression of neuroendocrine differentiation markers such as *IGF2*, TH, and CgA, and development of cellular processes with endocrine features and neuropil (48, 62, 63). This neuronal to neuroendocrine differentiation is referred to as chromaffin differentiation. (The word *chromaffin* comes from the Greek *chroma*, color, and the Latin *affinis*, having affinity for; cells staining strongly with chromium salts.) Neuroblastoma differentiation and apoptosis are therefore important regarding both prognostication and new treatment strategies.

CgA is an acidic monomeric protein which is co-stored and co-released by exocytosis from catecholamine storage vesicles in neural, neuroendocrine, and endocrine cells (64). During the characterization of our animal model, we found that small tumors, also had areas of hemorrhage and cellular debris. We therefore sought for a marker that would reflect the viable tumor cell population by being produced in and secreted by the tumor cells. We found that the plasma concentrations of CgA were directly proportional to the tumor burden and rose in parallel with tumor growth (44). This was in contrast to the clinically used neuroblastoma markers NSE and pancreastatin in plasma, and HVA, VMA, and dopamine in urine. Although plasma NSE and urinary HVA were elevated in tumor-bearing animals, only plasma CgA correlated with the tumor burden (44). This makes CgA a promising biochemical marker for neuroblastomas. The properties of CgA as a tumor marker need to be further evaluated in a clinical setting, however.



Fumagillin **TNP-470**

Fig. 4. Structure of fumagillin and its synthetic derivative, TNP-470.

Angiostatic drugs and treatment

The first angiostatic agent used in our studies was TNP-470 (Takeda Neoplastic Product, also known as angiogenesis modulator, AGM-1470). TNP-470 (65) was a kind gift of Takeda Chemical Industries Ltd., Osaka, Japan. It is an analogue of the epoxide fumagillin, a naturally secreted antibiotic of the fungus Aspergillus fumigatus (Fig. 4). TNP-470 is 50 times as potent as the naturally occurring substance fumagillin. TNP-470 inhibits endothelial cell proliferation and migration at ~10 pg/ml, far below (around 3 logs lower) its cytotoxic concentrations (41, 65, 66). The effect of TNP-470 on tumor growth has been studied in a variety of experimental tumors (66). Phase I-III clinical trials of TNP-470 are now in progress, mainly in the USA (Table 2). In these trials the drug is given intravenously for 1 to 4 hours every 2-3 days. Its dose-limiting symptoms of toxicity are reversible confusion, headache, and ataxia.

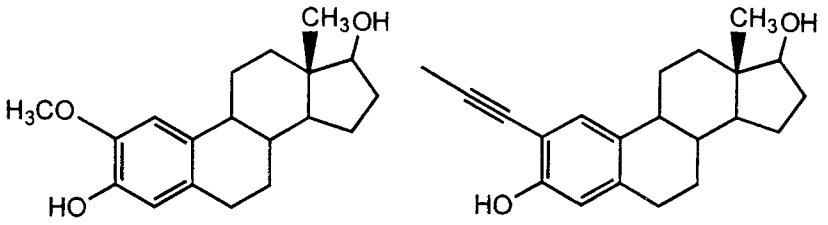
One group of animals was treated with TNP-470, and the other group served as a control. The effects were evaluated by measurements of tumor volume and analysis by transmission electron microscopy, scanning electron microscopy of microvascular casts, quantitative light microscopy, and immunohistochemistry (41, 45). We reported for the first time a tumoristatic effect of an angiogenesis inhibitor on human neuroblastoma. Not only a significantly reduced tumor growth rate, but also a reduced microvascular density and a reduction of the fraction of viable tumor cells, was observed in the TNP-470 treated animals (41, 45). Our results were confirmed in a recent study by Nagabuchi et al. (67), who showed that TNP-470 improved animal survival, and reduced tumor growth of primary and metastatic murine neuroblastoma. The plasma concentrations of TNP-470 do not reach cytotoxic levels, and thus we were able to document a tumoristatic effect of a single agent that did not act on the tumor cells directly but on growth-stimulated endothelial cells (41, 45).

When we analyzed the mechanisms underlying the tumor growth reduction induced by TNP-470 (45), we found that the apoptotic fraction of tumor cells increased more than threefold. In contrast,

the mean vascular diameter and the mean tumor cell proliferative index were unaffected. When differentiation markers were added to the protocol, it was seen that TNP-470-treated tumors exhibited striking chromaffin differentiation of neuroblastoma cells, observed as increased expression of *IGF2*, TH, and CgA, and increased formation of cellular processes. Statistical analysis revealed an inverse correlation between differentiation and angiogenesis. Morphological analysis revealed a gradient from proliferating over differentiating to apoptotic neuroblastoma cells with increasing distance from the feeding blood vessels. We suggest that by inhibiting angiogenesis, TNP-470 induces metabolic stress, resulting in chromaffin differentiation and apoptosis of neuroblastoma cells. This particular differentiation, designated agonal differentiation seems to be the link between angiostatic therapy and tumor cell apoptosis.

To see whether this phenomenon was a general pattern of angiostatic therapy in neuroblastoma, we decided to study other angiostatic drugs than TNP-470. Interest fell on the endogenous estrogen metabolite $2ME_2$, which inhibits angiogenesis and suppresses murine tumor growth (68, 69). Unlike the angiostatic steroids of corticoid structure (70), $2ME_2$ does not require co-administration of heparin or sulphated cyclodextrins for activity (68). Further, this metabolite had been shown to inhibit the growth of proliferating cancer cells (71). The mechanism for the bimodal and antimitotic activity of $2ME_2$ is not fully known, several pathways such as the interaction with tubulin, the induction of p53, and the impairment and imbalance of cell cycle kinases, have been reported as plausible (68, 69, 71).

During collaboration with EntreMed Inc. (Rockville, MD, USA), we received from them $2ME_2$ and a new synthetic analogue $2PE_2$, which has similar properties (Fig. 5). Because of the limited amounts of drugs available, the neuroblastoma cells were this time xenotransplanted to nude mice (46). Both drugs were given orally and potently suppressed neuroblastoma growth without signs



2-Methoxyestradiol **2-Propynylestradiol**

Fig. 5. Structure of $2ME_2$ and its derivative, $2PE_2$.

Table IV. A summary of the effects of 2 weeks of treatment with TNP-470, $2ME_2$, and $2PE_2$ on angiogenesis, tumor dynamics and differentiation. TNP-470: 10 mg/kg subcutaneously every other day, nude rats. $2ME_2$: 75 mg/kg orally twice a day, nude mice. $2PE_2$: 75 mg/kg orally twice a day, nude mice.

Angiostatic agent:	TNP-470	$2ME_2$	$2PE_2$
Tumor volume	- 66%	- 68%	- 74%
Viable tissue	- 33%	unchanged	- 31%
Angiogenesis *	- 36%	- 46%	- 44%
Proliferative cells	unchanged	unchanged	unchanged
Apoptotic cells	+ 219%	+ 225%	+ 283%
IGF2 expression	+ 88%	+ 140%	+ 124%
TH-positive cells	+ 96%	+ 139%	+ 196%

** Length of vessels per tumor volume (45, 46).*

of toxicity. By stereological quantification of tumor angiogenesis and tumor cell dynamics we demonstrated that both steroids exert their tumoristatic effect by inhibiting tumor angiogenesis and inducing tumor cell apoptosis. Hence, the action of these steroids is bimodal: both angiostatic and chemotherapeutic. These two steroids are of particular interest since they are active when administered orally. A striking chromaffin differentiation was also observed in the $2ME_2$- and $2PE_2$-treated tumors. Statistical analysis again revealed an inverse correlation of tumor angiogenesis to chromaffin differentiation and apoptosis. In view of their oral availability and potent antitumor effects, the angiostatic and chemotherapeutic steroids $2ME_2$ and $2PE_2$ are new candidates for clinical trials.

A summary of the effects of TNP-470, $2ME_2$, and $2PE_2$ on angiogenesis, tumor dynamics, and differentiation in neuroblastoma is given in Table IV.

CONCLUSIONS

- The animal experimental model is relevant and yields reproducible results (41, 44-46).
- The xenografted tumors grow exponentially and invasively in the host, retain their neuroblast phenotype, but does not metastasize (41, 44-46).
- Plasma levels of CgA are directly proportional to the tumor burden in neuroblastoma (44).
- Quantification of the fraction of viable tumor cells in treated tumors, can reveal that a tumoristatic effect is even better than an effect on measured tumor volume (45, 46).

- Even small tumors display areas of necrosis, hemorrhages, and apoptosis (45, 46).
- Stereological quantification of vascular parameters can be performed on perfused vessels, appearing as punched-out holes in sections (45, 46).
- Treatment with 10 mg/kg of TNP-470 every other day results in a reduced growth rate and a T/C quotient (mean volume of treated tumors / mean volume of control tumors) of 0.34 after 12 days in nude rats (41).
- A TNP-470 dosage of 20 or 30 mg/kg results in a rapid loss of body weight by more than 25% in nude rats (41).
- TNP-470-treatment reduces angiogenesis. The tumor cell apoptotic fraction increases more than threefold, while the fraction of viable tumor cells is decreased. The mean vascular diameter and the mean tumor cell proliferative index are unaffected (45).
- Treatment with 75 mg/kg of $2ME_2$ or $2PE_2$, twice a day, results in a reduced growth rate and a T/C quotient of 0.32 and 0.28 respectively after 14 days in nude mice (46).
- $2ME_2$ and $2PE_2$ are active when administered orally and whithout signs of toxicity (46).
- $2PE_2$-treatment reduces the fraction of viable tumor cells (46).
- $2ME_2$ and $2PE_2$ have a dual action, that is they exert their tumoristatic effect by inhibiting tumor angiogenesis and inducing tumor cell apoptosis. The mean vascular diameter and the mean tumor cell proliferative index are unaffected (46).
- Apoptosis is increased between twofold and fourfold in a dose-dependent manner, by $2ME_2$-treatment (46).
- $2ME_2$ and $2PE_2$ induce apoptosis of neuroblastoma cells in vitro (46).
- Neither TNP-470, $2ME_2$, nor $2PE_2$ has an effect on chromaffin differentiation of neuroblastoma cells in vitro (45, 46).
- Treated tumors (i.e. TNP-470, $2ME_2$, $2PE_2$) display a sleeve-like arrangement of neuroblastoma cells surrounding a central vessel, forming perivascular cuffs some 10-15 cell layers thick (41, 45, 46).
- A pattern with a sudden cellular differentiation at an approximate distance of 100 μm from the central vessel followed by apoptosis in the cuff periphery is evident in tumors treated with angiostatic agents (45, 46).
- Tumor angiogenesis is invertely correlated to chromaffin differentiation and apoptosis (45, 46).
- Inhibition of angiogenesis induces chromaffin differentiation and apoptosis in neuroblastoma. Such agonal differentiation may be the link between angiostatic therapy and tumor cell apoptosis (45, 46).
- Angiostatic agents administered as single therapy have an objective tumoristatic effect in our neuroblastoma model (41, 45, 46).

FUTURE PERSPECTIVES

There have been speculations as to the future clinical value of angiostatic agents in pediatric oncology (3). Hopefully our studies may provide an impetus to clinical trials of angiogenesis inhibitors at least in high-risk neuroblastoma patients who do not respond to conventional treatment. Today there are two ongoing clinical trials, one at the Memorial Sloan-Kettering Cancer Center, New York (NY, USA) and one at the Dana-Farber Cancer Institute, Boston (MA, USA): a phase I study of TNP-470 in patients with recurrent or refractory pediatric solid tumors, lymphomas, and acute leukemias. As yet there are no data on efficacy and side effects, but angiostatic agents have taken the step from experimental studies to clinical trials also in children with cancer.

New angiostatic agents are being developed. Among these the endogenous angiogenesis inhibitors are of particular interest, since they are a part of the normal regulation of vascular growth, and may have fewer side effects than exogenous ones. One endogenous inhibitor, angiostatin (18), is an internal fragment of plasminogen. Another, endostatin (19), is a cleavage fragment of collagen XVIII, and a third one, vasculostatin, is being characterized (Yuen Shing, personal communication). Preliminary in vitro and in vivo data indicate that the relaxed form of antithrombin III and an internal osteonectin fragment also are potent endogenous inhibitors of angiogenesis (Judah Folkman, personal communication). Clinically, it has been known that some patients exhibit growth of metastases within a few months after removal of a primary tumor. This phenomenon led to the discovery of angiostatin in mice. However, whether patients with advanced primary tumors without detectable metastases elaborate endogenous inhibitors of angiogenesis is not known. It has been speculated that angiogenesis is regulated by more than twenty endogenous protein fragments, in analogy with blood coagulation (7). One way to investigate this would be by testing serum and urine samples from patients with advanced but localized tumors on endothelial cell cultures. We are currently undertaking such an investigation.

Angiostatic therapy in combination with other treatment modalities is another field of research. Several reports of synergistic effects have been published, for example by Teicher et al. (22). Our finding that inhibition of angiogenesis induced chromaffin differentiation in neuroblastoma (45, 46), taken together with fact that neuroblastoma cells can be induced to differentiate in vitro (48), suggests that angiostatic therapy in combination with retinoic acid might be beneficial. Chromaffin differentiation also includes increased expression of CgA (45). CgA is normally secreted from highly vascularized neuroendocrine cells, and CgA and its cleavage fragments have some biological activity (64). Recently, it was reported that neuropeptide Y, a sympathetic cotransmitter

and regulatory peptide frequently produced by neuroblastomal cells, exerted angiogenic activity (72). We are therefore now examining whether CgA or its cleavage fragments may have an undiscovered angiogenic effect.

Another clinical application of angiogenesis research is monitoring of angiogenic peptides in urine or blood. These may be used in the future as biochemical markers of tumor angiogenesis. Every tumor type or even every tumor may have its own angiogenic profile, and by analyzing angiogenic peptides in body fluids it might be possible to optimize treatment with inhibitors that will have the best effect on that particular tumor. In our experimental model the neuroblastoma cells secrete bFGF and $VEGF_{165}$. Presently we are investigating the question of whether plasma or urine concentrations of these two angiogenic peptides correlate to the tumor burden in nude rats. Preliminary data in neuroblastoma patients show that circulating levels of VEGF, but not of bFGF, correlate to tumor stage and outcome (E Wassberg et al., in preparation). However, the reference ranges of angiogenic peptides are based on adults. For this reason we have initiated a prospective study to obtain a normal reference material for angiogenic peptides (i.e. angiogenin, bFGF, epithelial growth factor, hepatocyte growth factor, leptin, transforming growth factor β_1, tumor necrosis factor α, and VEGF) in healthy children. According to preliminary data, bFGF levels are much higher (6-8 times) in healthy young children than in healthy adults (E Sköldenberg et al., in preparation). This may indicate an upregulation of angiogenesis during normal growth, and stresses the importance of having appropriate reference values in children instead of extrapolating from levels in adults. Monitoring of angiogenic peptides may help the pediatric oncologist not only to predict a poor prognosis and a probability of metastasis, but also to assess the disease activity, detect relapses at an early stage (38), and identify cases where angiostatic agents are indicated.

In summary, the field of angiogenesis research has three important applications for treatment of patients with neuroblastoma: treatment with angiostatic agents, monitoring of angiogenic peptides to assess disease activity, and prediction of the outcome on the basis of microvascular counts. The clinical value of these applications remains to be settled, but the simplicity of the concept and its powerful therapeutic implications will be the driving forces of these future efforts.

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