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Review

Angiostatic Treatment of Neuroblastoma

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The growth of solid tumours 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. The mediators of tumour angiogenesis are now being elucidated, and angiostatic agents have been developed. Some of these agents are currently undergoing clinical trials. In addition to inhibition of angiogenesis, two other clinical applications of angiogenetic research in tumour diseases are monitoring of disease activity by analyses of circulating angiogenic peptides and prediction of a poor outcome by tumour microvascular counts. Neuroblastomas grow quickly, are highly vascularised and metastasise early and hence inhibition of angiogenesis—angiostatic therapy—may be indicated in this disease. The effects of treatment with the angiostatic agent TNP-470 in an experimental model results in a significant reduction of the tumour growth rate, reduced microvascular counts and a reduced fraction of viable tumour cells compared to controls. TNP-470 as single therapy has an objective tumoristatic effect in our neuroblastoma model. Angiostatic treatment of neuroblastoma is a new and theoretically promising treatment modality that merits clinical investigations. The feasibility of assessing disease activity by repeated determinations of the levels of circulating angiogenic peptides should also be determined, as well as the use of microvascular counts to predict a poor outcome. © **1997 Elsevier Science Ltd.**

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INTRODUCTION

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 healthy adult, however, angiogenesis is a rare event and vascular endothelial cells generally exhibit a slow turnover. More than two decades ago clinical observations and experimental studies led to the suggestion that tumour growth, just like the growth of any other tissue, is dependent on angiogenesis [1] and this has since been confirmed in a number of studies. Hence, a tumour does not rely on invasion by pre-existing vessels for expansion of the tumour cell population, but triggers the surrounding tissue to form new blood vessels to supply the tumour (Figure 1). There is now substantial evidence that tumour growth and metastasis are angiogenesis dependent (for reviews see [2–9]).

Angiogenesis is a tightly controlled and complex process involving several factors at the molecular and cellular levels, with both stimulating and inhibiting steps. The process by which malignant cells acquire their angiogenic phenotype and stimulate angiogenesis is now being unravelled [2, 4]. The angiogenesis cascade seems to be evolutionarily well conserved, although species differences have been reported. To date, 14 angiogenic peptides (Table 1) and several of their endothelial cell receptors have been identified. Apart from these angiogenic peptides, there are also agents of low molecular weight with angiogenic activity.

In a recent study it was found that the urine concentration of the angiogenic peptide basic fibroblast growth factor (bFGF) was frequently elevated in patients with a wide variety of human tumours [10]. The urine levels of bFGF were elevated at the time of diagnosis and decreased with treatment in childhood acute lymphoblastic leukaemia [11]. The

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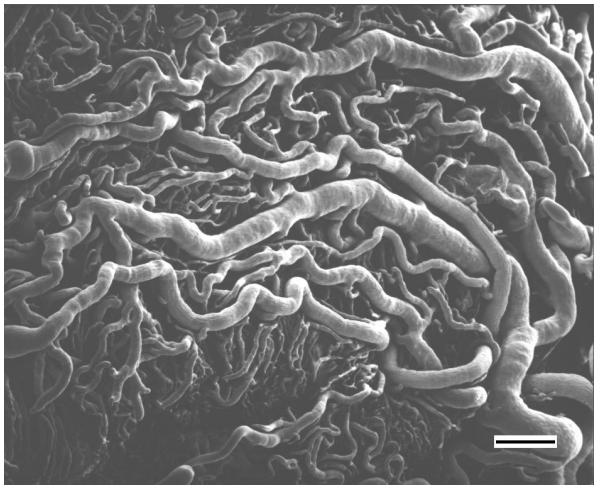

Figure 1. Human neuroblastoma xenotransplanted to nude rat. Microvascular corrosion cast at scanning electron microscopy. Overview of the tumour capsule. The casts are made by resin infusion through the thoracic aorta. The tumour microcirculation consists of newly formed sinusoidal tumour vessels, 10–80 μm in diameter, exhibiting extensive anastomoses. There are comparatively few true arteriovenous capillaries 4–10 μm in diameter. Scale bar = 500 μm.

latter phenomenon suggests that leukaemia cells induce angiogenesis in the bone marrow and leukaemia could therefore also be a candidate for treatment with angiostatic drugs. The urine levels of bFGF were also correlated to tumour stage and prognosis in Wilms' tumour [12]. As yet there are no reports on the urine levels of angiogenic peptides in neuroblastoma.

Tumour angiogenesis can be quantified by counting the number of microvessels per surface area in paraffin sections from the resected primary tumour after immunostaining with a suitable endothelial cell marker. Roughly 100 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 [5] for a review). In the only report on neuroblastoma [13], the microvascular count correlated with metastatic disease, *MYCN* amplification and a poor outcome.

Inhibitors of angiogenesis are now being identified increasingly rapidly [14]. Today, at least twelve angiostatic agents of different structures and with different modes of action have entered clinical trials (Table 2). Neuroblastomas grow quickly, are highly vascularised and metastasise early. Hence, it is likely that neuroblastoma elaborate angiogenic peptides and would respond to angiostatic treatment. Clinically, there is a need for more efficient treatment modalities in neuroblastoma, since the outcome is poor for a large group of patients [15]. Therefore, we investigated the response of experimental neuroblastoma to angiostatic agents (e.g. TNP-470).

Table 1. *Angiogenic peptides*

Angiogenic peptide	Molecular weight in kDa
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
Placental growth factor	25
Platelet-derived endothelial cell growth factor	45
Proliferin	35
Transforming growth factor α	5.5
Transforming growth factor β	25
Tumour necrosis factor α	17
Vascular endothelial growth factor	45

Except for angiopoietin-1 [29], complete citations are given in [2, 3].

RESULTS FROM OUR LABORATORY

The angiostatic agent used in our studies is TNP-470. It is a lipid, derived from the fungus *Aspergillus fumigatus*. It is 50 times as potent as the naturally occurring substance fumagillin. TNP-470 inhibits endothelial cell proliferation at approximately 10 pg/ml, far below its cytotoxic concentrations [16]. Phase I–III clinical trials of TNP-470 are now underway mainly in the U.S. (Table 2). The drug is given intravenously for 1 h every 2–3 days and so far has shown few side-effects.

To investigate the effects of angiostatic treatment in neuroblastoma, we developed a new animal experimental model. Details of the experimental procedures and the results are presented in detail elsewhere [17]. Cells from the poorly differentiated, adrenergic human neuroblastoma cell line SH-SY5Y [18] were used as tumour xenografts in nude rats. One group of animals were treated with TNP-470 and the other group served as controls. The angiostatic effects were evaluated by measurements of tumour volume and analysis by transmission electron microscopy, scanning electron microscopy of microvascular casts, quantitative light microscopy and immunohistochemistry.

We reported for the first time a tumoristatic effect of an angiogenesis inhibitor on human neuroblastoma. Not only a significantly reduced tumour growth rate, but also a reduced microvascular density and a reduction of the fraction of viable tumour cells were observed in the TNP-470 treated animals. It must be noted that these observations were made in an animal experimental setting with a xenografted monoclonal human neuroblastoma cell line which lacks *MYCN*

Table 2. *Angiogenesis inhibitors*

Agent	Clinical trial	Reference
Angiostatin	—	
Batimastat (BB94)	phase II	[30]
bFGF soluble receptor	—	
Carboxyamidotriazole	phase II	[31]
CM101	phase I	[32]
Endostatin	—	
Interferon α	phase III	[33]
Interleukin-12	phase II	[34]
Linomide	phase I	[35]
Marimastat (BB2516)	phase III	[30]
Platelet factor 4	phase II	[36]
Prolactin	—	
Suramin	phase II	[37]
Tecogalan (DS-4152)	phase I	[38]
Thalidomide	phase II	[39]
Thrombospondin-1	—	
Tissue inhibitors of metalloproteinases	—	
TNP-470	phase III	[40]

Except for [32, 37], complete citations are given in [2, 3]. For the latest update visit http://cancernet.nci.nih.gov/ (for clinical trials in U.S.A.) or http://telescan.nki.nl/ (for clinical trials in Europe).

amplification and does not exhibit metastatic growth. This model was chosen intentionally, since most clinical neuroblastomas lack *MYCN* amplification, and the absence of metastases facilitates accurate measurements of tumour size. The implications are nevertheless interesting: a tumoristatic effect of a single agent that did not act on the tumour cells directly but on growth-simulated endothelial cells. Also, in a recent study by Nagabuchi and associates [19] it was shown that TNP-470 treatment improved animal survival and reduced tumour growth of primary and metastatic murine neuroblastoma. There have been speculations as to the future clinical value of angiostatic agents in paediatric oncology [6], and hopefully these recent reports [17, 19] may provide an impetus for clinical trials of such agents in high-risk neuroblastoma patients who do not respond to conventional treatment.

POTENTIAL CLINICAL VALUE OF ANGIOSTATIC DRUGS IN NEUROBLASTOMA

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 possible side-effects—of these inhibitors are known, but it will also take even more time for the next generation of angiostatic agents (e.g. angiostatin [20] and endostatin [21]) to complete clinical trials. Angiostatin and endostatin have been demonstrated to induce tumour regression and tumour dormancy without drug resistance in several experimental models. In practice, however, angiostatic agents should perhaps be administered in combination with other therapies, since positive synergistic effects, both with cytotoxic agents and with radiotherapy, have been observed experimentally [7]. The theoretical mechanisms of these synergistic effects are that angiogenesis inhibitors increase the uptake of chemotherapeutic drugs into a tumour and increase the tumour blood flow, apparently by 'unpacking' tumour cells and by reducing interstitial pressure and hypoxia [7, 9, 22]. Also the combination of two or more angiogenesis inhibitors may be necessary to obtain a tumour response. Again, such phenomena will require detailed clinical investigations to be optimised. Inhibition of angiogenesis is a new strategy in treating patients with cancer, and merits thorough evaluation.

Potential toxicity

An anticipated side-effect of angiogenesis inhibition in paediatric patients is impairment of organ development and growth. Hopefully, angiostatic agents which specifically inhibit tumour-induced angiogenesis and not physiological angiogenesis will be developed in the future since there are promising candidate molecules that may be expressed only in tumour-induced blood vessels [23, 24]. In a critical perspective, however, most treatment modalities today impair growth, and certain chemotherapy regimens may even have serious long-term side-effects such as cardiotoxicity and secondary malignancies. Another drawback 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 [25]. There is a risk that dormant micrometastases [20, 26] may start to grow if angiogenesis inhibition is withdrawn too early. Further possible side-effects of angiostatic treatment could be impairment of wound healing and interference with the female reproductive system [27]. However, in situations in which wound healing is desired the treatment might be discontinued temporarily. 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 tumour angiogenesis.

Advantages for angiostatic therapy

Potential advantages with angiostatic therapy over chemotherapy are:

- Angiostatic therapy generally has low toxicity. It is directed mainly at proliferating capillary endothelial cells and does not cause bone marrow suppression, gastrointestinal symptoms or hair loss in patients (e.g. platelet factor 4 and TNP-470) [22]. Angiostatin and endostatin, for example, have shown no toxicity at all in animal models [20, 21].
- The development of drug resistance is less likely to occur, and has not been observed experimentally [2, 3, 9]. This is because the target cells are normal, untransformed endothelial cells, in contrast to tumour cells which are genetically unstable.
- The endothelial cells are by definition exposed to the circulation—in contrast to most tumour cells—and hence drug delivery is likely to be successful. Angiostatic agents do not have to cross the blood–brain barrier, for example.
- The metabolism of 10^4 tumour cells depends on one single capillary loop [28], so that even a limited reduction of the number of tumour vessels will affect a large number of tumour cells.

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

Angiogenesis research for prognostication

A potential clinical application of angiogenesis research is prognostication from vascular counts in resected tumour tissue. In well over 100 reports on different types of malignant tumours, including neuroblastoma, a significant correlation between high vascular counts and a poor prognosis has been found [8]. In our experimental setting, vessels could be identified by vascular perfusion fixation and quantified by an unbiased stereological method, proving the angiostatic effect of TNP-470.

Monitoring of angiogenic peptides in urine or blood may in the future be used as independent biochemical markers of tumour angiogenesis or aggressiveness. In our experimental model, the neuroblastoma cells secrete basic fibroblast growth factor and vascular endothelial growth factor 165. Presently we are investigating if plasma or urine concentrations of these two angiogenic peptides correlate with tumour burden in nude rats and with tumour stage and prognosis in patients. It is quite feasible that the vascular count in the pathologist's report and monitoring of angiogenic peptides may help the paediatric oncologist not only to predict poor prognosis and probability of metastasis, but also to assess disease activity, detect relapses early [11] and identify cases where angiostatic agents are indicated.

CONCLUSION

In summary, the field of angiogenesis research has three important applications in the treatment of patients with

neuroblastoma: treatment with angiostatic agents, monitoring of angiogenic peptides to assess the disease activity and predicting the outcome on the basis of microvascular counts. The clinical value of these applications still needs to be settled, but the simplicity of the concept and its powerful therapeutic implications will be the driving forces for these future efforts.

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