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Review

Angiogenesis and anti-angiogenesis in neuroblastoma

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Abstract

Angiogenesis is a biological process by which new capillaries are formed from pre-existing vessels. It occurs in physiological and pathological conditions, such as tumours, where a specific critical turning point is the transition from the avascular to the vascular phase. Tumour angiogenesis depends mainly on the release by neoplastic cells of growth factors specific for endothelial cells that able to stimulate the growth of the host's blood vessels. This review summarises the literature concerning the relationship between angiogenesis and progression in human neuroblastoma, the most common extracranial solid tumour of infancy and childhood. It is becoming increasingly evident that agents which interfere with blood vessel formation also block tumour progression. Accordingly, anti-angiogenic tumour therapy has gained much interest in preclinical and clinical assessments. The recent applications of anti-angiogenic agents which interfere or block neuroblastoma progression are reviewed. © 2002 Elsevier Science Ltd. All rights reserved.

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1. Angiogenesis and tumour angiogenesis

Angiogenesis, the biological process by which new capillaries are formed from pre-existing vessels, is a complex cascade that passes from basement membrane degradation, endothelial cell migration and invasion of the extracellular matrix, to endothelial cell proliferation and capillary lumen formation. The newly formed microvasculature is then stabilised by investment of the vessel wall with pericytes and subsequent inhibition of endothelial proliferation, basement membrane reconstitution, and junctional complex formation [1]. Several assays for studying angiogenesis are available (Table 1). Angiogenesis occurs in both physiological and pathological conditions (Tables 2 and 3). In solid tumour growth, a specific clinical turning point is the transition from the avascular to the vascular phase [2]. Once it has developed an intrinsic vascular network, the tumour grows indefinitely (unlike other forms of angiogenesis, tumour angiogenesis is not limited in time) both *in situ* and at distant sites (metastasis) since this enables its cells to enter the vascular bed and colonise other organs [3]. Tumour angiogenesis depends mainly on the release by neoplastic cells of growth factors that are specific for endothelial cells (Table 4) and that stimulate the growth of the host's blood vessels [4]. Substantial laboratory and indirect clinical evidences of the central role of angiogenesis in the progression of bladder, brain, breast, cervical, colon, lung, prostate and testis tumours, and haematological malignancies, including acute myeloid leukaemia, acute lymphoblastic leukaemia and multiple myeloma has been obtained in the last 20 years [4].

2. Biological and clinical aspects of neuroblastoma

Neuroblastoma, together with lymphoma, osteosarcoma, Ewing's tumours, rhabdomyosarcoma and lymphoblastic leukaemia, belong to a group of undifferentiated paediatric malignancies known as the small round-cell tumours of childhood. Neuroblastoma is the most common extracranial solid tumour of infancy and childhood [5]. It arises from primitive neuroepithelial

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Table 1
Models of angiogenesis

In vitro
Endothelial cell culture system
Ex vivo
Human placental blood vessels fragments in fibrin gel
Rat aorta explants
In vivo
Corneal micropocket model
Chick embryo chorioallantoic membrane assay
Rat subcutaneous air sac model
Angiogenesis *in vivo* using basement membrane extracts (Matrigel)

cells of the neural crest and occurs in the adrenal medulla or paraspinal symphathetic ganglia of the abdomen, chest or neck. Tumour stage and patient age at diagnosis correlate strongly with survival [5–7]. Neuroblastoma can form relatively benign, localised and well-differentiated tumours that are successfully treated by surgical resection alone (stage I or II) or locally invasive (stage III) and metastatic (stage IV) tumours that are associated with a bad clinical outcome [8]. Distant metastases commonly occur in the regional lymph nodes, liver, bone marrow and bones [9]. However, in children less than one year of age, metastases limited to the bone marrow, liver or skin, but absent from the bones (stage IV S) are associated with a favourable outcome [5]. In some patients, the tumour may regress spontaneously, via differentiation into benign ganglioneuroma [10]. This differentiation has always been viewed as evidence that neuroblastoma exhibits a sympathetic neuronal phenotype. Chromaffin differentiation along a fetal-specific extra-adrenal lineage is now known to be common in neuroblastoma areas with focal apoptosis [11]. A specific marker for this type of differentiation is the expression of the gene encoding insulin-like growth factor II (*IGF-2*). For children older than 1 year with metastatic disease, the outcome is usually fatal. This wide range of clinical variability reflects neuroblastoma's biological heterogeneity. Numerous studies have demonstrated that the molecular and cytogenetic features of clinically aggressive neuroblastoma differ from those observed in tumours associated with a good response to therapy. Biological variables such as histopathology [12,13] (favourable features include the presence of an abundant schwannian stroma, evidence of neuroblastic differentiation), cellular DNA content or chromosome number [14], N-*myc* copy number [15,16], deletion of the short arm of chromosome 1 [17,18], and nerve growth factor receptor (*TRK-A*) mRNA expression [19–22] are of prognostic significance.

Table 2
Physiological angiogenesis

Ovulation
Development of the corpus luteum
Embryogenesis
Lactating breast
Immune response
Wound repair

Table 3
Pathological angiogenesis

Neoplasia
Solid and haematological tumours
Cardiovascular disorders
Atherosclerosis
Haemangiomatosis
Ocular disorders
Diabetic retinopathy
Neovascular glaucoma
Retrolental fibroplasia
Chronic inflammatory diseases
Diabetes
Psoriasis
Pyogenic granuloma
Rheumatoid arthritis
Systemic sclerosis

3. Angiogenesis in neuroblastoma

In 1994, Kleinman and colleagues showed that human neuroblastoma cells induce angiogenesis in the nude mouse during tumorigenesis [23]. Two days after intradermal injection, many small blood vessels, branched towards the tumour cell mass from larger dermal blood vessels in the vicinity of the injection site. After 1 week,

Table 4
Proangiogenic growth factors

Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF)
Placental growth factor (PlGF)
Basic fibroblast growth factor/fibroblast growth factor-2 (bFGF/FGF-2)
Acidic fibroblast growth factor/fibroblast growth factor-1 (aFGF/FGF-1)
Fibroblast growth factor-3 (FGF-3)
Fibroblast growth factor-4 (FGF-4)
Transforming growth factor-α (TGF-α)
Transforming growth factor-β (TGF-β)
Epidermal growth factor (EGF)
Hepatocyte growth factor/scatter factor (HGF/SF)
Tumour necrosis factor-α (TNF-α)
Platelet-derived growth factor (PDGF)
Granulocyte colony-stimulating factor (G-CSF)
Erythropoietin (Epo)
Interleukin-8 (IL-8)
Pleiotropin
Thymidine phosphorylase/platelet-derived endothelial cell growth factor (TP/PD-ECGF)
Angiogenin

these vessels had enlarged, while those growing in the opposite direction had disappeared. Meitar and colleagues [24] found that the vascularity of primary untreated neuroblastoma in patients with widely metastatic disease is significantly higher in patients with local or regional disease.

We have investigated two human neuroblastoma cell lines, LAN-5 and GI-LI-N to induce both human microvascular endothelial cells *in vitro* to proliferate and angiogenesis *in vivo* in the chick embryo chorioallantoic membrane (CAM) assay [25]. Conditioned medium (CM) from LAN-5 stimulated *in vitro* endothelial cell proliferation more than GI-LI-N CM. Moreover, anti-vascular endothelial growth factor (VEGF), but not anti-fibroblast growth factor-2 (FGF-2) antibodies, prevented this proliferation. LAN-5 cells induced angiogenesis to a greater extent than GI-LI-N cells in the CAM assay.

Canete and colleagues [26] used an anti-CD34 antibody to evaluate tumour angiogenesis in a retrospective study of 69 patients. Vascularity was not significantly correlated with other prognostic factors, such as age, stage, histopathology, TRK-A, P-glycoprotein expression, or N-*myc* copy number, nor with the occurrence of relapses, or survival. They concluded that tumour vascularity is not predictive of survival and that neither disseminated nor local relapses are influenced by the angiogenic characteristics of the tumours.

4. High level expression of angiogenic factors is associated with advanced tumour stage in human neuroblastoma

Melster and colleagues [27] analysed the expression of VEGF in six human neuroblastoma cell lines and five primary neuroblastoma. High VEGF levels were found in the supernatant of all cell lines and in tissue homogenates from four primary tumours; all cell lines and primary tumours expressed the VEGF receptor Flk-1, though neutralising antibodies to VEGF did not inhibit cell growth.

Rossler and colleagues [28] demonstrated that VEGF is expressed and secreted by neuroblastoma cells and upregulated by hypoxia.

Langer and colleagues [29] studied VEGF, vascular endothelial growth factor receptor-1 (*VEGFR-1*) and *VEGFR-2* mRNA expression in neuroblastoma surgical specimens and cell lines by reverse transcriptase-polymerase chain reaction (RT-PCR). All lines expressed VEGF mRNA, whereas only the SK-N-BE line expressed mRNA coding for the VEGRs. The specimens expressed both VEGF and VEGRs mRNA.

A systematic analysis of the expression of angiogenic factors in 22 neuroblastoma cell lines and in 37 tumour samples by Eggert and colleagues [30] showed that high expression of seven factors (VEGF-$A_{165/121}$, VEGF-B, VEGF-C, FGF-2, Angiopoietin 2 (Ang-2), transforming growth factor-α (TGF-α) and platelet-derived growth factor-A (PDGF-A) was strongly correlated with advanced stage neuroblastoma. This suggest that several angiogenic peptides act in concert in the regulation of neovascularisation. A significant positive correlation was demonstrated between the expression of PDGF-A alone and overall survival.

5. Matrix metalloproteinase expression in human neuroblastoma

Additional events involved in tumour progression comprise secretion of matrix-degrading enzymes by tumour cells, including two matrix metalloproteinases (MMPs), namely type IV, 72 kDa (MMP-2 or gelatinase A) and 92 kDa (MMP-9 or gelatinase B) collagenases. MMP-2 and MMP-9 facilitate invasion and metastasis due to degradation of type IV, V, VII and X collagens as well as fibronectin [31], important constituents of the interstitial stroma and subendothelial basement membrane. In human colon, breast and lung carcinoma and melanoma, MMP-2 [32] and MMP-9 [33] are markedly overexpressed during the invasive and metastatic phases, while they are almost or completely absent in hyperplastic or normal tissue and *in situ* tumours.

Immunohistochemical evaluation of the relationship between the expression pattern of MMP-2, MMP-9 and their specific inhibitor TIMP-2 with clinical variables in 31 patients with neuroblastoma by Ara and colleagues [34] showed that increased expression of MMP-2 in stromal tissues was significantly associated with advanced clinical stages. TIMP-2 staining was mostly confined to the neoplastic cell cytoplasm of stromal tissue and endothelial cells, and decreased TIMP-2 expression was also significantly related to advanced disease.

Sugiura and colleagues [35] examined the expression of MMP-3 and its corresponding tissue inhibitors (TIMP-3) in seven human neuroblastoma cell lines and 24 primary untreated tumours. MMP-2 was detected predominantly in an inactive proform in all cell lines and tumour tissue extracts. The lack of MMP-2 activation in the cell lines was attributed to the absence of expression of a membrane-type MMP (MT1-MMP) which activates proMMP-2, and to the abundant expression of TIMPs, particularly TIMP-2. Immunohistochemical analysis of tumour tissue samples indicated that MMP-2 was present in both the tumours and stromal cells. In contrast, MMP-9 was not expressed by the neuroblastoma cell lines, but was present in both inactive and active forms in extracts from tumour tissues. Immunohistochemical analysis of positive specimens indicated that it was predominantly present in stromal, vascular and perivascular cells surrounding nests of tumour cells. There was no correlation between

the levels of these MMPs and the N-*myc* copy number or the histopathological phenotype. However, higher levels of MMP-2 and MMP-9 were observed in stage IV than in stages I and II.

Sarakibara and colleagues [36] demonstrated that higher ratios of gelatinase activation resulting from high expression of a new membrane-type matrix metalloproteinase-1 (MT-MMP-1) on neuroblastoma specimens, is significantly associated with advanced stage and unfavourable outcome.

We have studied two human neuroblastoma cell lines, LAN-5 and GI-LI-N to see if they secrete MMP-2 and MMP-9 [25]. Both lines secreted the active form of MMP-2 almost exclusively. We have also investigated tissues from biopsies of human neuroblastoma immunohistochemically with an antibody against factor VIII in order to determine their microvessel number, and by *in situ* hybridisation to determine the expression of MMP-2 and MMP-9 [37]. Results showed that the extent of angiogenesis and the MMP-2 and MMP-9 expression were upregulated in advanced stages.

6. Amplification of N-*myc* is associated with enhanced angiogenesis of human neuroblastoma

N-*myc* is a nuclear transcription factor expressed during the development of the central nervous system, spinal ganglia, lungs and kidney [38]. It appears to be necessary for normal cardiovascular development, since N-*myc* knockout mice die *in utero* [39] or suffer from serious cardiopulmonary defects [40]. N-*myc* may also regulate the growth of neuroblastoma vessels, by its amplification or overexpression associated with angiogenesis in experimental [41] and clinical settings [24]. Amplification of N-*myc* is a frequent event in advanced stages of human neuroblastoma (III and IV) and correlates with poor prognosis and enhanced vascularisation, suggesting that the N-*myc* oncogene could stimulate tumour angiogenesis and thereby allow disease progression.

7. Vascular integrin α_v β_3: a new prognostic indicator in neuroblastoma

Angiogenesis is also influenced by receptors for extracellular matrix proteins. Cell adhesion to the extracellular matrix is mediated by integrins, a family of heterodimeric transmembrane proteins that comprise over 15 α and 8 β subunits. Previous studies [42–44] demonstrated that integrin α_v β_3 plays a key role in angiogenesis induced by a variety of *in vivo* stimuli and promotes vascular endothelial cell survival *in vivo* [45].

Erdreich-Epstein and colleagues [46] demonstrated by immunohistochemical analysis that $\alpha v\beta_3$ integrin was expressed by 61% of microvessels in high-risk neuroblastoma (stage IV and N-*myc* amplified), but only by 18% of microvessels in low-risk tumours (stage I and II and non-N-*myc* amplified).

Table 5
Mechanism of action of anti-angiogenic compounds

Inhibitors of extracellular matrix remodelling
Inhibitors of adhesion molecules
Inhibitors of activated endothelial cells
Inhibitors of angiogenic mediators or their mediators
Inhibitors of endothelial cell intracellular signalling

8. Anti-angiogenesis in neuroblastoma

The existence of specific angiogenesis inhibitors was first postulated by Folkman in 1971 [47]. The term 'anti-angiogenesis' was introduced to describe treatment designed to prevent the induction of new blood vessels and perhaps reduce the number of those already present. Inhibitors of angiogenesis are grouped as class 1 (specific and semi-specific) and class 2 (non-specific), depending on whether they only inhibit proliferation and/or migration of endothelial cells or are also cytotoxic for tumour cells (Table 5) [48]. Over 20 anti-angiogenic drugs are currently undergoing evaluation in phase I, II or III clinical trials (Table 6).

TNP-470 (or AGM-1470), a class 1 inhibitor, is a synthetic derivative of fumagillin that inhibits methionine aminopeptidase-2, a cytoplasmic enzyme of endothelial cells [49], cell proliferation [50] and cell migration [51], thus blocking capillarogenesis *in vitro* [52,53] and angiogenesis *in vivo* [54–57]. It is currently being applied in phase II or III trials for the treatment of solid tumours [58,59].

The genetic instability and high mutation rate of tumour cells are partly responsible for the frequent emergence of acquired drug resistance with conventional cytotoxic anticancer therapy, whereas endothelial cells are not likely to acquire such resistance [60]. A number of current anti-angiogenic clinical trials have been designed to compare the effects of a particular chemotherapeutic agent alone (Table 7) with those of its combination with an anti-angiogenic inhibitor. The combination of TNP-470 with a conventional cytotoxic agents, such as cisplatin, paclitaxel or cyclophosphamide, significantly improves their antitumour efficacy [61].

Cells from the poorly differentiated human neuroblastoma cell line SHSY5Y were used as tumour xenografts in nude rats [62]. One group of animals were treated with TNP-470 and the other group served as controls. Treatment with TNP-470 induced a reduction in the tumour growth rate, microvascular density and the fraction of viable tumour cells. Nagabuchi and colleagues [61] showed that TNP-470 improves animal survival and reduces the growth of primary and meta-

Table 6
Angiogenesis inhibitors in clinical trials

Drug name	Trial
Marimastat[a]	Phase III
AG 3340[a]	Phase III
Metastat[a]	Phase I
BMS-2752291[a]	Phase I
Neovastat[a]	Phase I/II
CGS 27023A[a]	Phase I/II
Bay 12-9566[a]	Phase III
EMD 121974[c]	Phase II/III
Vitaxin[c]	Phase II
SV 5416	Phase I/II
PTK 787/ZK22584[d]	Phase I
Purlytin (SnET2)[e]	Phase I/II/III
Suradista (suramine derivate)	Phase I/II
Su 101[f]	Phase II/III
Flavopiridol	Phase I
TNP-470	Phase II
Thalidomide	Phase III
ZD0101 (CM 101)[g]	Phase II
Combrestatin A4	Phase I
CAI[b]	Phase II
Squalamine[h]	Phase I/II
Paclitaxel (Taxol)	Phase I/II
Su 6668[i]	Phase I
Endostatin[j]	Phase I
Interleukin-12	Phase I/II
Interferon-alpha	Phase II/III
CT-2584[k]	Phase II
IM-862[l]	Phase III
BeneFin[m]	Phase II/III

[a] MMP inhibitors.
[b] CAI corresponds to carboxyamide-triazole.
[c] Antagonists of $\alpha v\beta 3$ integrin.
[d] Block VEGF receptor signaling.
[e] Is a photoreactive purpurine.
[f] Blocks PDGF receptor signaling.
[g] A bacterial derived polysaccharide.
[h] An aminoesterol.
[i] Blocks VEGF, FGF and PDGF receptors signaling.
[j] A collagen XVIII fragment.
[k] A xantine analogue.
[l] A dipeptide.
[m] Purified stark cartilage protein.

Table 7
Chemotherapeutics agents with reported antiangiogenic activity

Alkylators
Cyclophosphamide
Edelfosine
Estramustine
Melphalan
Antimetabolites
5-fluorouracil
Methotrexate
Mercaptopurine
UFT
Tegafur
Uracil
Cytarabine
Antitumour antibiotics
Bleomycin
Daunorubicin
Daxorubicin
Epirubicin
Mitomycin
Mitoxantrone
Topoisomerase inhibitors
Camptothecin
Irinotecan
Etoposide
Topotecan
Taxanes
Docetaxel
Paclitaxel
Vinca alkaloids
Vinblastine
Vincristine

static murine neuroblastoma. To investigate whether TNP-470 inhibits neuroblastoma growth more effectively in animals with a low tumour burden Katzenstein and colleagues [63] used it to treat 30 nude mice with minimal disease. Treatment was initiated before the tumour burden was clinically apparent, either 12 h or 1 week after subcutaneous inoculation with cells from the human neuroblastoma cell line NBL-W-N. These authors also treated animals 3–9 weeks after tumour cell inoculation, when small (<400 mm^3) or large (>400 mm^3) tumours had developed. They demonstrated that TNP-470 inhibits neuroblastoma growth when administered in the setting of animal disease. Indeed, TNP-470 reduces the growth rate of small tumours, but does not significantly alter that of large tumours. Wassberg and colleagues [63] demonstrated that TNP-470 reduces tumour growth and increases the tumour cell apoptotic fraction. Moreover, TNP-470-treated tumours exhibited striking chromaffin differentiation of neuroblastoma cells. The authors suggested that by inhibiting angiogenesis, TNP-470 induced metabolic stress, resulting in chromaffin differentiation and apoptosis. Shusterman and colleagues [64], showed that TNP-470 significantly inhibited tumorigenicity when administered shortly after neuroblastoma xenograft inoculation and following cyclophosphamide.

We have used the CAM assay to study the effects of the synthetic retinoid fenretinide (HPR) on certain endothelial cell functions *in vitro* and *in vivo* [65]. Results showed that HPR inhibited VEGF- and FGF-2-induced endothelial cell proliferation without affecting endothelial motility; and inhibited growth factor-induced angiogenesis in the CAM assay. A significant anti-angiogenic potential of HPR has been also observed with respect to the angiogenesis induced *in vivo* by neuroblastoma biopsies. We previously demonstrated that supernatants derived from neuroblastoma cell lines stimulate endothelial cell proliferation [25] and that this effect is abolished when neuroblastoma cells

are incubated in the presence of HPR. VEGF- and FGF-2-specific enzyme-linked immunosorbent assays (ELISA), performed on both CM derived from neuroblastoma cells cellular extracts, indicated no effect of HPR on the level of these angiogenic cytokines. Moreover, RT-PCR analysis of *VEGF* and *FGF-2* gene expression confirmed this lack of effect. HPR also significantly repressed the spontaneous growth of endothelial cells. Lastly, immunohistochemical experiments performed in the CAM assay demonstrated that endothelial staining of both VEGF receptor-2 and FGF-2 receptor-2 was reduced after implantation of HPR-loaded sponges compared with the control CAM. These data suggest that HPR exerts its anti-angiogenic activity through both a direct effect on the endothelial cell proliferative activity and an inhibition of the response of endothelial cells to the proliferative stimuli mediated by the angiogenic growth factors.

9. Concluding remarks

Understanding of the basic biology of angiogenesis and tumour biology has recently led to the development of novel strategies for the treatment of cancer patients. The complex relationship between the angiogenic cascade and anti-angiogenic agents in the tumour vascular phase, as well as the identification and characterisation of angiogenesis inhibitors, has indicated that anti-angiogenesis may be considered for the adjuvant therapy of neuroblastoma.

A major goal is the determination of whether inhibition of angiogenesis is a realistic way of inhibiting tumour cell dissemination and the formation of metastasis in neuroblastoma. Long-term, regular low-dose administration of chemotherapeutics agents could inhibit endothelial cell proliferation, angiogenesis, and subsequent tumour growth. We have recently demonstrated, using the CAM assay, that extremely low (e.g. picomolar) doses of vinblastine devoid of endothelial cytotoxicity, can still block aspects of angiogenesis *in vitro* and *in vivo* [66]. Klement and colleagues [67] have subjected two neuroblastoma cell lines to either continuous treatment with low doses of vinblastine, the monoclonal anti-flk-1 neutralising antibody called DC 101, or both agents together. Both DC 101 and low-dose vinblastine treatment individually resulted in significant, but ultimately transient, xenograft regression and diminished tumour vascularity. Remarkably, the combination therapy resulted in full and sustained regression of large established tumours, without an ensuing increase in host toxicity or any signs of acquired drug resistance during the course of treatment, which lasted more than 6 months. Similarly, camptothecin, topecan and paclitaxel at low and non-cytotoxic concentrations can block endothelial functions [68,69]. These results raise the important question of the optimal low-dose of a given chemotherapeutic drug that is required to induce an anti-angiogenic effect [70,71].

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