Pergamon

European Journal of Cancer, Vol. 35, No. 3, pp. 445–449, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(98)00387-6

Original Paper

Expression of Vascular Endothelial Growth Factor (VEGF) and its Receptors in Human Neuroblastoma

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Angiogenic factors may play a role in the biology of neuroblastoma, a well vascularised tumour, which frequently spreads haematogenously. Therefore, we analysed expression of vascular endothelial growth factor (VEGF) in six human neuroblastoma cell lines and five primary neuroblastomas. High VEGF levels (1–3 ng/10^6 cells/day) were found in the supernatant of all cell lines examined (SK-N-LO, SK-N-SH, LS, SH-SY5Y, IMR-32, Kelly). VEGF peptide was also detected in tissue homogenates from four of five primary tumours. Reverse transcription–polymerase chain reaction (RT–PCR) revealed that $VEGF_{165}$ is the major isoform produced by neuroblastomas. In addition, all cell lines and primary tumours expressed the mitogenic VEGF receptor FLK-1, whilst the non-mitogenic receptor FLT-1 was less frequently positive, suggesting that the tyrosine kinase FLK-1 is involved in malignant transformation of neuroblastoma cells. However, neutralising antibodies to VEGF did not inhibit growth of neuroblastoma cell lines, which argues against a role of VEGF as an autocrine growth factor, at least for cell lines *in vitro*. We conclude that neuroblastoma cells produce VEGF, which may contribute to tumour vascularisation, growth and invasion.

Key words: VEGF, neuroblastoma, angiogenesis, FLK-1, FLT-1
Eur J Cancer, Vol. 35, No. 3, pp. 445–449, 1999

INTRODUCTION

NEUROBLASTOMA IS one of the most common solid tumours in children which frequently spreads haematogenously [1, 2]. Many clinical symptoms, particularly of the metastatic disease, are related to the fact that most primary neuroblastomas and metastases are well vascularised. Spontaneous haemorrhage into the tumour and manifestations leading to bleeding or thrombosis have also been reported [3]. Histopathological studies have demonstrated that vascular proliferations are characteristic features of neuroblastomas [4]. In addition to these specific characteristics, *in vivo* growth of malignant tumours beyond 1–2 mm in diameter generally depends critically on tumour vascularisation [5]. Thus, it is conceivable that angiogenic factors play a role in the biology of neuroblastoma.

Vascular endothelial growth factor (VEGF) is a major cytokine involved in the establishment of blood vessels during embryonic development as well as angiogenesis in non-malignant and malignant states [6]. A continuous supply of VEGF may be critical for the integrity of endothelium *in vivo* [7]. It has been demonstrated that overexpression of VEGF by malignant cells supports the development of tumour vasculature and contributes to the increased permeability of tumour vessels [8]. VEGF is a 34–42 kDa heparin-binding, dimeric, disulphide-bonded glycoprotein which exists in four isoforms (121, 165, 189 and 206 amino acids, respectively) [9]. The proliferative effect of VEGF on endothelial cells is mainly mediated by fetal liver kinase-1 (FLK-1, also termed KDR (kinase insert domain-containing receptor) or VEGF receptor-2) [10]. Other VEGF receptors (e.g. *fms*-like tyrosine kinase-1 (FLT-1), also termed VEGF receptor-1) [11, 12] modulate cellular functions such as cell migration, without being mitogenic [13]. While expression of FLK-1 in non-malignant cells is confined to the endothelium, FLT-1 is also expressed in other cell types, such as haematopoietic cells [13]. Expression of VEGF mRNA has been reported in a variety of human tumours including glioblastomas, gastrointestinal, ovarian, breast and prostate cancer [14–19].

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Received 22 Jun. 1998; revised 5 Oct. 1998; accepted 29 Oct. 1998.

We analysed the expression of VEGF mRNA and peptide in neuroblastoma cell lines and primary tumour cell specimens. In parallel, mRNA expression of FLK-1 and FLT-1 was assessed. Our results suggest that VEGF plays a role in the development of tumour vascularisation in human neuroblastoma. Consistent expression of FLK-1 suggests involvement of this tyrosine kinase in malignant transformation of human neuroblastoma.

MATERIAL AND METHODS

Cell lines

Human neuroblastoma cells SK-N-SH, SK-N-LO, SH-SY5Y, IMR-32, LS and Kelly were kindly provided by Dr R. Handgretinger (Department of Paediatrics, University of Tübingen, Germany). The cells were maintained in RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (PAA Laboratories, Linz, Austria), 1% sodium pyruvate, 1% essential amino acids, 1% non-essential amino acids and 1% penicillin–streptomycin (Gibco, Paisley, U.K.). To analyse the secretion of VEGF into the supernatant, cells were grown in serum-free medium (X-Vivo 10 with gentamicin, Bio Whittaker, Walkersville, Maryland, U.S.A.) for 24 h and the supernatant collected.

Patients and tumours

Primary neuroblastoma or ganglioneuroblastoma tumour specimens were obtained from previously untreated patients undergoing surgery. All patients were treated at the University Hospital of Innsbruck, Austria. Patient characteristics including stage of disease were classified according to the International Staging System [1], as shown in Table 1.

Preparation of cytosolic extracts

Tissue homogenates from tumour samples were prepared as described previously [20]. Before homogenisation, the specimens were pulverised in liquid nitrogen, diluted with four volumes of homogenisation buffer (10 mmol/l Tris–HCl, pH 7.4, containing 1.5 mmol/l ethylenediamine tetraacetic acid (EDTA), 5 mmol/l disodium molybdate, 100 ml/l glycerol and 1 mmol/l monothioglycerol) and centrifuged at 50 000 ***g*** for 30 min (4°C). The protein concentration of the cytosolic extracts was determined using the Biuret assay (Boehringer Mannheim, Mannheim, Germany). The mean protein concentration of neuroblastoma cytosolic extracts was 3.36 ± 0.1 g/dl (mean ± standard deviation (SD)). The VEGF concentration was measured by enzyme-linked immunosorbent assay (ELISA) as described below. The amount of VEGF was expressed as pg/g total protein.

ELISA for VEGF

The VEGF concentration in samples from conditioned medium was measured using a human VEGF immunoassay which recognises the soluble isoforms $VEGF_{121}$ and $VEGF_{165}$ (R&D Systems, Minneapolis, Minnesota, U.S.A.). For cell culture supernatants a sensitivity of 5 pg/ml could be achieved.

Reverse transcription (RT) and polymerase chain reaction (PCR)

Oligonucleotide primers spanning the insertion/deletion site of human $VEGF_{165}$ were synthesised. The sense primer was 5′-CGAAGTGGTGAAGTTCATGGATG-3′ and the antisense primer was 5′-TTCTGTATCAGTCTTTCCTGGTGAG-3′. RT–PCR of mRNA encoding the 121, 165 and 189 amino acid-containing isoforms of VEGF results in PCR products of 403, 535 and 607 bp length [7, 9]. Primers were synthesised for FLK-1 [7] (sense: 5′-CTGGCATGGTCTTCTGTGAAGCA-3′, antisense: 5′-AATACCAGTGGATGTGATGCGG-3′, expected size of the PCR product: 790 bp) and FLT-1 (sense: 5′-GTCACAGAAGAGGATGAAGGTGTCTA-3′, antisense: 5′-CACAGTCCGGCACGTAGGTGATT-3′, expected size of the PCR product: 417 bp). First strand cDNA was synthesised by RT of 200 ng total RNA isolated from the cells using guanidine thiocyanate and amplified by *Taq* DNA polymerase dissolved in PCR buffer (KlenTaq, Clontech, Palo Alto, California, U.S.A.) in a 50 µl reaction containing 0.2 mM dNTPs and 40 pmol of each primer. The PCR profile consisted of 1 min initial denaturation at 94°C, followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 62°C, 2 min polymerisation at 72°C and finally 10 min extension at 72°C. Twenty microlitres of the PCR products were separated in 2% wt/vol agarose gels and stained with ethidium bromide.

Blocking experiments with an antihuman VEGF neutralising antibody

Neuroblastoma cells (1×10^4) were seeded into 25 cm^2 culture flasks containing X-Vivo 10 medium supplemented with 5% heat-inactivated fetal calf serum. For neutralisation of human VEGF bioactivity, 1 µg/ml of a monoclonal antihuman VEGF antibody, which reacts with the soluble isoforms $VEGF_{121}$ and $VEGF_{165}$ (clone 26503.11, R&D Systems, Wiesbaden, Germany; half maximal inhibition of

Table 1. *Patient characteristics*

Patient	Sex	Age	Histology	Stage (INSS)	Primary tumour	Metastases	N-Myc amplification	Del (1 p)	Ploidy
1	M	3 years	Neuroblastoma	4	Thoracic	Lymph node, bone, bone marrow intracranial	+	+	Hyperdiploid
2	F	15 years	Neuroblastoma	4	Suprarenal	Lymph node, bone, bone marrow	+	+	Diploid
3	M	3 years	Ganglioneuroblastoma	2a	Thoracic	–	n.d.	n.d.	n.d.
4	F	1 month	Neuroblastoma	4	Suprarenal	Liver	–	–	Diploid/ tetraploid
5	F	3 years	Ganglioneuroblastoma	2a	Suprarenal	–	n.d.	–	n.d.

n.d., not determined; INSS, International Neuroblastoma Staging System.

VEGF bioactivity at 0.06 μg/ml, as measured by inhibition of proliferation of human umbilical vein endothelial cells) was added daily to SK-N-LO and SH-SY5Y neuroblastoma cells and 10 μg/ml to SK-N-LO, LS, SH-SY5Y and Kelly cells. For control experiments, 1 μg/ml or 10 μg/ml mouse IgG, whole molecule (Dianova, Hamburg, Germany) was added daily to the culture medium. Growth curves were established by counting the total cell number per flask every other day (in triplicate) until day 9.

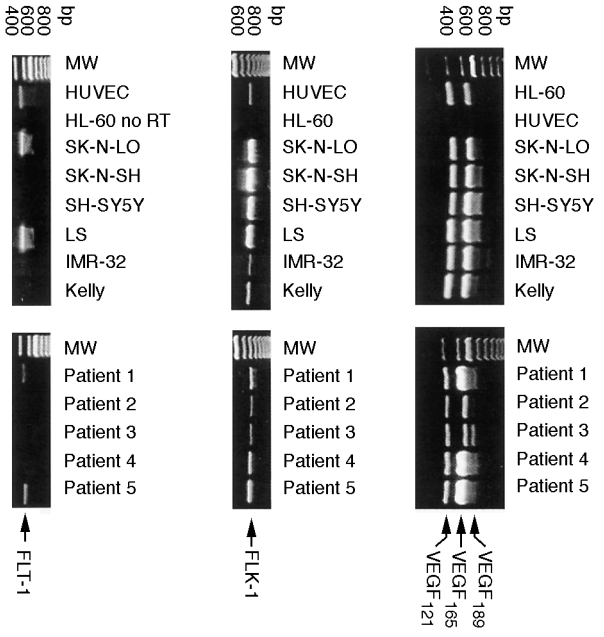

Figure 1. Vascular endothelial growth factor (VEGF), FLK-1 and FLT-1 mRNA expression in neuroblastoma cell lines and primary tumours. By reverse transcription–polymerase chain reaction (RT–PCR), all six tumour cell lines and five primary tumour cell specimens (patients 1–5) expressed mRNA of the three VEGF isoforms ($VEGF_{189}$, $VEGF_{165}$ and $VEGF_{121}$). The isoform $VEGF_{165}$ was most abundantly transcribed. RNA from the cell line HL-60 served as a positive control, RNA from human umbilical vein endothelial cells (HUVEC) as a negative control. All cell lines and primary tumour cell specimens simultaneously expressed FLK-1 mRNA. As controls, HUVEC was positive and HL-60 was negative for FLK-1. In contrast, FLT-1 mRNA was only found in some tumour cell lines and primary tumour samples. HUVEC served as a positive control, HL-60 without RT (no RT) as a negative control.

RESULTS

VEGF and VEGF receptor mRNA expression by human neuroblastoma cell lines

By RT–PCR, all six neuroblastoma cell lines expressed VEGF mRNA. As shown in Figure 1, the isoform $VEGF_{165}$ was most abundantly transcribed in all cell lines. In contrast, the band corresponding to $VEGF_{121}$ appeared to be less intense and only weak expression of $VEGF_{189}$ was detected. Whilst FLT-1 mRNA was found only in SK-N-LO, LS and weakly in IMR-32 cells, expression of FLK-1 mRNA was consistently detected in all neuroblastoma cell lines.

VEGF and VEGF receptor mRNA expression in primary human neuroblastomas

By RT–PCR, VEGF mRNA was found in the tumour tissue from all 5 neuroblastoma patients (Figure 1). Similar to the results from the cell lines, $VEGF_{165}$ was the predominant isoform at the mRNA level, particularly in the samples from patients 1, 4 and 5. The results of RT–PCR analysis for VEGF receptor expression also corresponded to the results from the cell lines. Whilst FLK-1 mRNA was consistently expressed in all tumour samples, FLT-1 mRNA was only found in some tumour samples (patients 1 and 5).

VEGF secretion by neuroblastoma cell lines and primary tumours

Using a sensitive ELISA assay, large amounts of VEGF peptide were detected in the supernatant of all six neuroblastoma cell lines examined (Figure 2a). All cell lines produced between 1 and 3 ng VEGF/10^6 cells during an incubation period of 24 h.

Significant levels of VEGF were detected in the tissue homogenates from four of five primary neuroblastomas (Figure 2b). Interestingly, the samples with the greatest VEGF concentration (patients 1, 4 and 5) also showed the

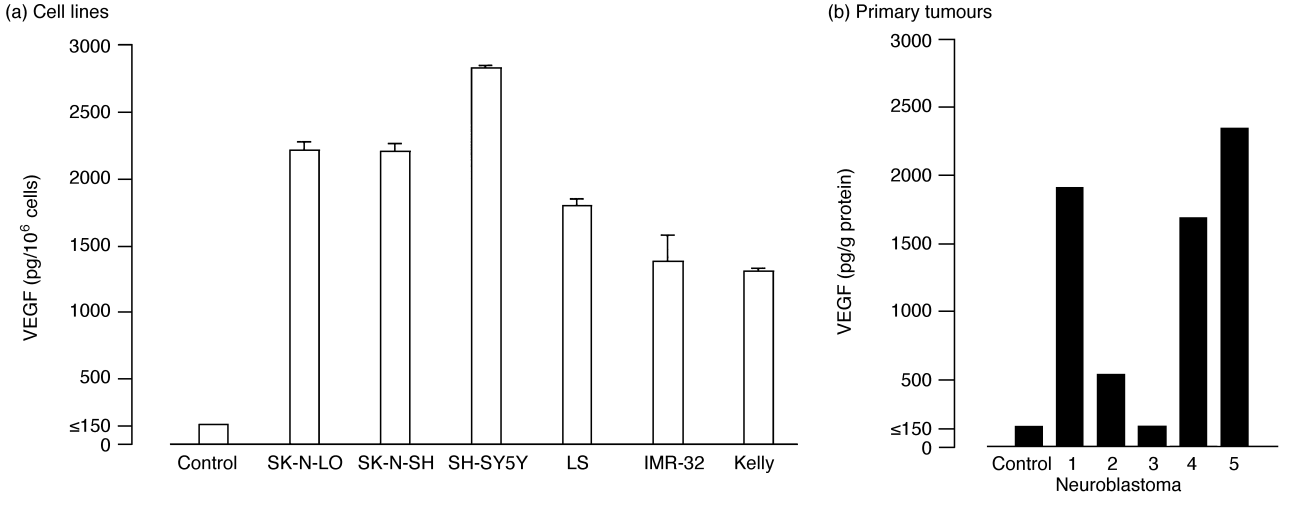

Figure 2. Vascular endothelial growth factor (VEGF) concentrations in cell culture supernates and primary tumour specimens. (a) During 24 h incubation in serum-free medium, the human neuroblastoma cell lines SK-N-LO, SK-N-SH, LS, SH-SY5Y, IMR-32 and Kelly released VEGF into the supernatant. Medium not conditioned by cells served as a negative control (Control). (b) Significant amounts of VEGF were also detected in tissue homogenates from four of five primary neuroblastomas. Homogenisation buffer served as a negative control (Control).

strongest expression of $VEGF_{165}$ by RT–PCR. Taken together, these results indicate that $VEGF_{165}$ is the predominant isoform synthesised and secreted by neuroblastomas *in vivo*.

Blocking experiments with a monoclonal antihuman VEGF neutralising antibody

To assess whether blocking of VEGF activity by a neutralising monoclonal antibody results in growth inhibition of neuroblastoma cells, we performed experiments with four different neuroblastoma cell lines (SK-N-LO, LS, SH-SY5Y and Kelly). Two different antibody concentrations (1 and 10 μg/ml) were used. In all experiments, the addition of neutralising antibody to VEGF did not result in inhibition of proliferation of neuroblastoma cells (data not shown).

DISCUSSION

In this study, we demonstrated that human neuroblastoma cell lines and primary tumours express the angiogenic cytokine VEGF both at the protein and mRNA level. In addition, the mitogenic VEGF receptor FLK-1 was expressed in all cell lines and primary tumour specimens, whilst the non-mitogenic VEGF receptor FLT-1 was inconsistently positive. Expression of VEGF in neuroblastoma fits well with the previously described vascular proliferation associated with this tumour entity [4]. In general, macroscopic growth of malignant tumours requires the establishment of tumour vasculature [5], which by itself could explain the detection of the angiogenic cytokine VEGF in human neuroblastoma. In addition, neuroblastoma cells have previously been shown to produce a factor that supports endothelial cell growth [21]. According to our results, this finding is most likely due to secretion of VEGF. A recent study also demonstrated the *in vivo* angiogenic capacity of conditioned medium from two neuroblastoma cell lines which could be blocked by anti-VEGF antibodies, without directly analysing VEGF production by the tumour cells [22]. In addition, it has been reported that angiogenesis and vascularisation correlate with poor prognosis in neuroblastoma [23]. In our study, we demonstrated that VEGF is frequently produced by neuroblastoma cells *in vivo* and *in vitro*. However, further studies, including long-term follow-up of patients with neuroblastoma, are required to determine whether VEGF expression in primary tumours is of prognostic significance. Similar to other human malignancies [14–19], VEGF is likely to play a major role in the establishment of vascularisation in neuroblastoma.

Interestingly, not only VEGF itself, but also the mitogenic VEGF receptor FLK-1 was consistently expressed in neuroblastoma cells, whilst the non-mitogenic receptor FLT-1 was less frequently detected. In contrast, in normal tissues, expression of FLK-1 is more restricted than expression of FLT-1 [10–13]. Provided that VEGF is present in the tumour tissue, acquisition of FLT-1 by a VEGF-secreting tumour cell would not result in a growth advantage as compared with acquisition of FLK-1. One might speculate that co-expression of VEGF and FLK-1 in neuroblastoma cells leads to an autocrine loop supporting malignant cell growth. However, we did not detect growth inhibition of neuroblastoma cell lines *in vitro* when high concentrations of a neutralising VEGF antibody were added to the cultures, which argues against this hypothesis. Since most cells from malignant tumours do not spontaneously grow *in vitro*, growth factor requirements of cell lines *in vitro*, which represent selected clones, may differ from the primary tumours. Thus, the results of *in vitro* experiments using cell lines do not exclude the possibility that VEGF acts as a paracrine growth factor for primary neuroblastoma cells *in vivo*. However, addition of neutralising VEGF antibody was reported to have no effect on the *in vitro* growth rate of rhabdomyosarcoma, glioblastoma and leiomyosarcoma cell lines, *in vivo* treatment of nude mice with anti-VEGF antibody inhibited the growth of xenografted tumours and simultaneously decreased the density of tumour vessels [24]. Taken together, these findings suggest that stimulation of tumour vascularisation is the main effect of tumour cell-derived VEGF in human neuroblastoma, rather than acting as an autocrine growth factor.

Since the existence of an autocrine loop (VEGF and FLK-1 expressed by the same neuroblastoma cell) seems to be unlikely, the significance of FLK-1 expression by neuroblastoma cells is unclear. One might speculate that similar to other tyrosine kinase receptors, such as the Met tyrosine kinase and the epidermal growth factor receptor (EGFR), activating mutations could contribute to the transformed phenotype of the tumour cell [25, 26]. However, mutations of the FLK-1 tyrosine kinase have not been reported in human tumours so far.

In addition to FLK-1 and FLT-1, other VEGF receptors, which are expressed by malignant tumours, have recently been described. Particularly, neuropilin-1 has been identified as a specific receptor for $VEGF_{165}$, which enhances the binding of $VEGF_{165}$ to FLK-1 and is involved in vascular development [27, 28]. Further studies are required to evaluate the role of this receptor which might be expressed particularly in malignant tumours of the nervous system and modulate the effect of VEGF on tumour and endothelial cells. The fact that FLK-1 interacts with other cell surface receptors suggests a regulatory network controlling VEGF binding and signalling which is only partially understood. Thus, further characterisation of mechanisms regulating FLK-1 function may also have implications for the elusive role of neuroblastoma-associated FLK-1 expression.

The use of dose-intensified cytotoxic therapy has resulted in improved intermediate disease control in neuroblastoma [1, 2]. Despite the considerable initial response rates, the overall survival for the high risk group has remained essentially unchanged for the last three decades [1, 2]. Given the fact that angiogenesis is a key step in tumour growth, invasion and metastasis, and that our results suggest that VEGF plays a role in tumour angiogenesis in human neuroblastomas, blocking VEGF (or its receptors) could be an effective therapeutic approach. Since the main target of this cytokine appears to be the tumour vasculature rather than the tumour cells themselves, this could be a future treatment option for neuroblastoma, in addition to chemotherapy and radiation, which both act primarily on the tumour cells.

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Acknowledgements—We thank Alexandra Schüller and Petra Mayer for expert technical assistance.