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Original paper

COMPARATIVE ANALYSIS OF EXPRESSION OF ANGIOGENIC FACTORS AND CD44 GENE IN HUMAN GLIOMA AND NEUROBLASTOMA CELL LINES *IN VITRO*

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Summary: Angiogenesis is essential for tumor growth and relies on the production of angiogenic factors. By comparative analysis using RT-PCR method of angiogenic growth factors: VEGF, bFGF, PDGF-A, angiogenin-1 and IL-8 we established the level of expression of these genes necessary for angiogenesis in glioma and neuroblastoma cell lines. Our analyses were also extended to CD44 gene, which plays an important role in cascade of metastasis and progression of brain tumors. Significant differences in the level of gene expression of angiogenic factors and CD44 gene between the two cell lines observed throughout this study can be used as a prognostic marker for predicting clinical outcome in human brain tumors at the time of the initial staging.

Key words: angiogenic growth factors, CD44 gene, RT-PCR, glioma, neuroblastoma

Introduction

Tumors of the central nervous system (CNS) constitute a small fraction of the overall human cancers. However, their incidence is continually increasing and among children they take the second place just after leukemia. Gliomas represent a group of neoplasms derived from neuroepithelial tissue. Histologically, they are classified into four groups ranging from I (benign) to IV (glioblastoma) and the treatment depends on pathological diagnosis. Glioma arises in all age groups, however, low-grade tumors occur predominantly in children and high-grade ones in elderly people. Despite being characterized by a high invasive activity, glioma is rarely metastatic outside the CNS, indicating a successful regional prognosis and treatment of the patients (1–2).

Neuroblastoma (NB) is the most frequent solid extracranial malignancy in children and a major cause of death from cancer in infancy. It consists of neural crest derived from undifferentiated neuroectodermal cells. Tumor stage and patient age at diagnosis correlate strongly with survival. NB can form relatively benign, localized and well-differentiated tumors that are successfully treated by surgical resection alone (stage I or II) or locally invasive (stage III) and metastatic (stage IV) tumors associated with a fatal clinical outcome. Many NB present at diagnosis with metastatic disease are usually associated with poor survival despite intensive therapy (3–4).

Angiogenesis is essential for tumor growth and relies on the production of angiogenic factors. It has become generally accepted that solid tumors have to develop a vascular system for nutrient delivery and waste removal in order to grow appreciably (5). This process, angiogenesis, is critical to the progression of brain tumors, with vascular changes accompanying the advancement of these tumors. The cascade of events in this process of blood vessel formation involves a complex interplay between tumor cells, endothelial cells and their surrounding basement membranes (6–7).

In the present study, the contribution of angiogenic growth factors-vascular endothelial growth fac-

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tor (VEGF165), basic fibroblast growth factors (bFGF), platelet-derived growth factor-A (PDGF-A), angiogenin-1 (Ang-1) and interleucin-8 (IL-8) to glioma and NB was examined. These growth factors may influence glioma and NB angiogenesis by directly stimulating endothelial cell proliferation, by mediating the expression of key proteases on endothelial cells necessary for angiogenesis, or by regulating the expression of VEGF and other relevant genes. Although, the biological mechanisms that underlie the clinical variability observed in glioma and NB including tumor stage, patient age, histology, molecular markers, metastatic insemination etc., it has been shown that angiogenesis remains largely unknown in the regulation of brain tumors (8 11).

In this work, we extended our analyses to demonstrate the correlation between the expression of CD44 gene and that of different angiogenic factors involved in tumor angiogenesis and development of metastatic phenotype. The cell adhesion glycoprotein CD44 is a polymorphic molecule resulting from alternative splicing. It plays an important role in cascade of metastasis and progression of human malignant tumors. The analysis of CD44 gene expression in human malignant tissues has been shown to display a strong association with striking differences between metastatic and non-metastatic prognostic value (12 14).

The aim of this study was to develop a rapid method using RT-PCR for detection of different angiogenic factors and CD44 gene expression as a prognostic marker for predicting clinical outcome in human brain tumors at the time of initial staging. Moreover, the understanding of the basic mechanisms involved in brain tumor angigenesis could lead to development of angiogenesis-inhibiting factors as novel anti-tumor agents with a number of advantages over conventional chemotherapeutics.

Material and Methods

Cells and culture conditions

Human glioma cell lines U-87 MG (HTB-14) and neuroblastoma SK-N-SH (HTB-11) were obtained from American Type Culture Collection (ATCC, Rockville, U.S.A.) and only low passage cell lines were used. Both cell lines were maintained as monolayer cultures in Eagle's Minimal Essential medium with Earle's BSS and 2 mmol/L L-glutamine EMEM containing 10% fetal bovine serum (Sigma, Germany) and 1 mmol/L sodium pyruvate, 0.1 mmol/L nonessential amino acids, 1.5 g/L sodium bicarbonate (Sigma, Germany) at 37 °C and 5% CO_2 95% air-atmosphere in humidified incubator. The cells harvested in trypsin/EDTA solution in PBS and double washed in PBS were ready for the isolation of total RNA.

RNA isolation and reverse transcription (RT)

Total RNA was isolated from 5×10^6 cells by Qiagen RNAeasy total RNA preparation kit (Germany) according to the manufacturer's instruction. After purification and washing, the RNA was eluted with 50 μL DEPC-treated water. The purity and yield of the total RNA were determined spectrophotometrically and its integrity checked by agarose gel electrophoresis.

For the synthesis of cDNAs, 1 μg of total RNA was reverse transcribed in RT buffer (containing 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, 5 mmol/L $MgCl_2$), 100 mmol/L DTT, 2.5 μmol/L Oligo $(dT)_{16}$, dNTPs 0.5 mmol/L each, 1 U RNase inhibitor and 2.5 U murine leukemia virus reverse transcriptase (MuLV RT) in a final volume of 10 mL (Gibco-BRL, U.S.A). The reaction cycle consisted of 1 h at 42 °C, 10 min at 95 °C and cooling to 5 °C. The cDNAs were kept at 20 °C. To minimize errors in pipetting among the samples, master mixes of cDNA synthesis buffer containing dNTPs, RT buffer, oligo(dT), DTT and the enzymes were prepared and used for RT of all RNA samples in experiment.

PCR assay

For PCR amplification, appropriate dilutions of cDNA samples representing 100 200 ng of total RNA were mixed with PCR buffer containing 100 mmol/L dNTPs, 1.5 mmol/L $MgCl_2$, 10 × Stoffel Buffer (containing 50 mmol/L KCl and 10 mmol/L Tris-HCl), 0.8 1 μmol/L each of the primers and 1.25 U Stoffel *Taq* polymerase in a total volume of 25 mL (Perkin-Elmer, U.S.A).

A PCR primer pair for angiogenic growth factors and CD44 sequences obtained from Gene Bank, were used to design the primer pairs. All oligonucleotide primer pairs spanned the intron/exon splice site, ensuring that PCR products did not arise from DNA contamination present in the RNA preparations. The amplification products were normalized against the »housekeeping« gene GAPDH and cyclophilin (p1B15). The primer sequences and PCR product size are presented in *Table I* (15).

For the CD44 and IL-8 gene amplification cycle parameter was as follows: 55 °C for 8 min, 30 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and the final cycle 72 °C for 8 min. For the rest of the samples: VEGF, bFGF, PDGF-A and angiogenin-1 cycle parameters in all cases were: 94 °C for 1 min, 61 °C for 2 min and 72 °C for 2 min (30 cycles). PCR-amplified products were analyzed by electrophoresis in 1.5% agarose gel. All PCRs were done at least five times. The levels of CD44 mRNA were compared and a correction was made for differences in GAPDH mRNA. The levels of angiogenic growth factors mRNAs were compared and corrected for p1B15 mRNA levels. The expression of the target gene was

Table I Primer Sequences and Annealing Temperatures Used

cDNA	Primer Sequence	Ta (°C)	PCR product (bp)
VEGF165 Sense Antisense	5'-GGATGACTATCAGCGCAGCTAC-3' 5'-TCACCGCCTCGGCTTGTCACATC-3'	61	454
Ang-1 Sense Antisense	5'-CATCATGAGGAGACGGGG-3' 5'-TCCAAGTGGACAGGTAAGCC-3'	61	264
bFGF Sense Antisense	5'-ATGGCAGCCGGGAGCATCACC-3' 5'-CACACACTCCTTTGATAGACACAA-3'	61	239
PDGF-A Sense Antisense	5'-GAGTGAGGATTCTTTGGACACC-3' 5'-CTTCTTCCTGACGTATTCCACC-3'	61	322
IL-8 Sense Antisense	5'-TTGGCAGCCTTCCTGATTTC-3' 5'-AACTTCTCCACAACCCTCCTG-3'	55	247
CD44 Sense Antisense	5'-GGATCCATGAGTGGTATGGGA-3' 5'-GAATTCACCGACAGCACAGACAGA-3'	55	480
P1B15 Sense Antisense	5'-AGAAGCGCATGAGCATTGTGGAAG-3' 5'-TGCTCTCCTGAGCTACAGAAGGAA-3'	61	159
GAPDH Sense Antisense	5'-CGGAGTCAACGGATTTGGTCGTAT-3' 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'	55	306

normalized by taking the ratio of the densitometric unit of the gene/densitometric unit of internal control, p1B15 or GAPDH. For the PCRs, relative signal intensities were calculated and the data presented as the mean SEM.

Results

Since SK-N-SH and U-87 MG were shown to secrete high amounts of VEGF (16 17), we selected these tumor cell lines to study the level of expression of some angiogenic factors such as: $VEGF_{165}$, bFGF, PDGF-A, IL-8 and Ang-1. In this study we also investigated CD44 gene expression to confirm their metastatic potential in tumorogensis of glioma and NB.

To determine which angiogenic factors were expressed predominantly in SK-N-SH and U-87 MG, we analyzed the expression of the angiogenic mRNA in human glioma and NB cell lines using semiquantitative RT-PCR. Representative examples of RT-PCR products were separated by agarose gel electrophoresis presented in *Figure 1*. It can be seen that mRNA of all angiogenic factors and CD44 gene in SK-N-SH and U-87 MG cell lines were detected by this method. Quantification of mRNA expression was performed by densitometric analysis of the gels and taking the ratio of target gene and housekeeping gene as internal control-normalized target gene. The results are summa-

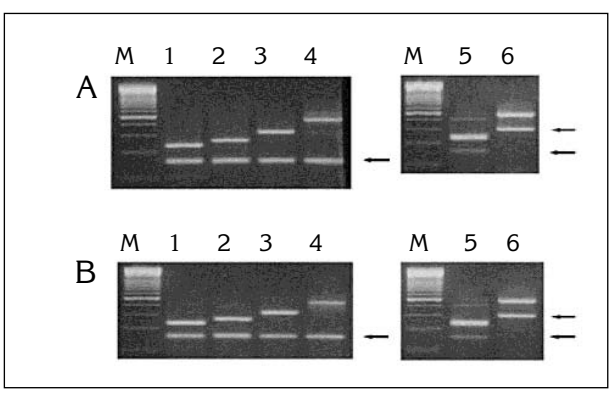

Figure 1. Expression of VEGF165, bFGF, PDGF-A, Ang-1, IL-8 and CD44 genes in human neuroblastoma SK-N-SH (A) and glioma U-87 MG (B) cell lines. The expression of angiogenic factors and CD44 was determined by RT-PCR. Total RNA was extracted from confluent cultures as described under Materials and methods. The first strand cDNA was synthesized by reverse transcription of total cellular RNA and PCR-amplified with varying primer concentrations. RT-PCR products were subjected to electrophoresis in 1.5 % agarose gel, stained with ethidium bromide and photographed under UV light. Quantification was performed using Multi-Analyst/PC Software Image Analysis System (BIO-Rad, Gel Doc 1000). Panel A, neuroblastoma, SK-N-MC. Lanes: 1 bFGF; 2 Ang-1; 3 PDGF-A; 4 VEGF165; 5 IL-8 and 6 CD44. Panel B, glioma, U-87 MG. Lanes: Lanes: 1 bFGF; 2 Ang-1; 3 PDGF-A; 4 - VEGF165; 5 IL-8 and 6 CD44. M molecular size markers. Arrows indicate the position of housekeeping genes.

Table II Mean values of angiogenic factors and CD44 epression in neuroblastoma cell line SK-N-SH

	n	$\bar{x}$	δ	CV (%)
VEGF	3	± 0.339	0.177	13%
Ang-1	3	± 0.259	0.011	1%
bFGF	3	± 0.355	0.146	14%
PDGF	3	± 0.404	0.329	27%
IL-8	3	± 1.683	0.461	9%
CD44	3	± 0.197	0.146	25%

The expression of the target gene was normalized by taking the ratio of the densitometric unit of the gene/densitometric unit of internal control, p1b15 or GAPDH.

Table III Mean values of angiogenic factors and CD44 expression in glioma cell line U-87 MG

	n	$\bar{x}$	δ	CV(%)
VEGF	3	0.264	0.190	18%
Ang-1	3	0.431	0.352	20%
bFGF	3	0.828	0.560	23%
PDGF	3	2.045	1.131	18%
IL-8	3	8.456	8.770	35%
CD44	3	0.313	0.228	24%

The expression of the target gene was normalized by taking the ratio of the densitometric unit of the gene/densitometric unit of internal control, p1b15 or GAPDH.

rized in *Tables II* and *III*. As shown in *Table III*, in U-87 MG cell line $VEGF_{165}$, Ang-1, bFGF, PDGF-A, IL-8 and CD44 were upregulated from 1.0 19-times more than in SK-N-SH cell line *(Table II)*. In this cell line, the most prominent level of IL-8 mRNA expression was detected. Although, the level of expression of bFGF, PDGF-a and IL-8 mRNA was also highly upregulated in SK-N-SH cell line, the difference between the two cell lines was obvious and the expression of these genes was from 3 19-fold increased in U-87 MG cell line.

Discussion

Angiogenesis is essential for tumor growth and metastases formation. Numerous angiogenic factors that regulate this complex process alone or in synergy have been identified. So, VEGF, bFGF, PDGF, Ang-1, IL-8 *etc*. have been shown to induce angiogenesis in a variety of experimental models (10, 18 19).

Our results indicate that all five angiogenic factors examined here showed a broad expression pattern in human glioma and NB cell lines suggesting their involvement in angiogenesis of brain tumors (20 21). VEGF is an important angiogenic factor and endothelium-specific mitogen, which has been implicated in the neovascularization of a wide variety of tumors. It acts via a paracrine mechanism mainly through two specific receptors at the surface of endothelial cells (22 24). In our study both cell lines exhibited a significantly low VEGF mRNA expression in comparison with the other angiogenic factors. These results are in agreement with our previous report where we demonstrated that human glioma cell lines responded to EGF with a higher stimulation of VEGF secretion into the culture medium comparing to those growing in EGF-free medium (16). For this reason, we concluded that this result is in accordance with our present finding demonstrating that VEGF165 mRNA positively correlates with its protein expression as an indicator of VEGF secretion of these cells without EGF stimulation.

The results obtained throughout the present study clearly demonstrate that bFGF mRNA indicates a relatively higher level of expression (3.8-fold) comparing to VEGF mRNA in glioma cell lines as determined by RT-PCR assay. The identification bFGF mRNA in glioma cell lines tested is consistent with a recent report that increased expression of bFGF is associated with malignant progression of astrocytic tumors (25). Relative expression of bFGF, as well as the level and type of receptor expression may be key determinants of the transformed state in tumors of glial origin (1).

Angiogenin (Ang-1), the ligand for TIE-2, a receptor-like tyrosine kinase expressed in endothelial cells, seems to be important to maintain blood vessels integrity by mediating interactions between the endothelium and surrounding matrix (26). Since it stabilizes the structure of newly-formed blood vessels, it takes part in the process of angiogenesis later than VEGF. Expression levels of VEGF gene family, bFGF and Ang-1 are correlated with each other in glioma and NB tumors and besides, with a high degree of vascularization suggesting their synergistic action in tumor angiogenesis.

In the present study, we demonstrated several time higher level of expression of IL-8 and PDGF-A mRNAs in U-87 MG glioma cell lines than in SK-N-SH *in vitro*. We found that such high expression levels both of IL-8 and PDGF-A mRNAs correlated strongly with advanced stage of malignancy. Their different levels of expression in the two cell lines used in this work suggest that different signal transduction pathways are included in the regulation of angiogenesis at different levels (17).

PDGF consists of two related polypeptides (α- and β-chain) and it binds to two classes of the receptors α- and β-receptors Endothelial cells may respond to autocrine and paracrine PDGF-B through their β-receptors whereas the a-receptors on glioma cells proper may be activated by autocrine PDGF-A. It was originally known to be involved in the regulation of cell migration and proliferation, but more recently was

reported to possess an angiogenic capability both *in vitro* and *in vivo* (27). Our results demonstrated here that the expression of PDGF-A was highly elevated in both examined cell lines, especially in glioma, suggesting that in glioma cells PFGF-A may play an angiogenic or cell migrating rather than mitogenic role.

In the present study, we found that the CD44 standard form was expressed in a relatively low level comparing to the other angiogenic factors. Moderate to low CD44 expression was detected in these two cell lines indicating that CD44 expression did not appear to be directly related to amplification and overexpression of the oncogenes, but rather to the morphological cell type and lineage (28).

In conclusion, further studies are necessary to understand in detail biological significance of angiogenesis inhibitors and activators, which directly affect vascularity, tumor growth and metastasis. Taken together, our results suggest that several angiogenic factors play biological role in angiogenesis. They might contribute synergistically to a more aggressive angiogenic process targeting molecules for antiangiogenic therapy providing novel strategy that may be particularly useful for highly metastasic advanced stage tumors (29 30). More general antiangiogenic approaches using synthetic small molecules, to inhibit endothelial proliferation independent on angiogenic factor expression, might be more promising in brain tumors therapy.

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UPOREDNA ANALIZA EKSPRESIJE ANGIOGENIH FAKTORA I CD44 GENA U HUMANIM ĆELIJSKIM LINIJAMA GLIOMA I NEUROBLASTOMA *IN VITRO*

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Kratak sadržaj: Angiogeneza je neophodna za rast tumora i zahteva proizvodnju angiogenih trofičkih faktora koji učestvuju u tumorogenezi. Uporednom analizom angiogenih trofičkih faktora: VEGF, bFGF, PDGF-A, angiogenina-1 i IL-8 pomoću metode RT-PCR utvrdili smo nivo ekspresije ovih gena uključenih u proces angiogeneze u ćelijskim linijama glioma i neuroblastoma. Takođe smo proširili analize i na CD44 gen koji igra važnu ulogu u kaskadi nastanka i progresiji metastaza tumora mozga. Dobijeni rezultati ukazuju na značajnu razliku u nivou genske ekspresije angiogenih faktora i CD44 gena u ove dve ćelijske linije čije se poreklo razlikuje ne samo po nastanku već i po mestu rasejavnja metastaza. Rezultati bi mogli da posluže kao prognostički faktor u preklinički i kliničkim istraživanjima tumora mozga od inicijalnih do terminalnih stupnjeva nastanka i terapije.

Ključne reči: angiogeni faktori rasta, CD44 gen, RT-PCR, glioma, neuroblastoma, ekspresija gena

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