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Research Paper

Production and Post-Surgical Modification of VEGF, tPA and PAI-1 in Patients with Glioma

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KEY WORDS

VEGF, tPA, PAI-1, lp (a), high grade glioma, thromboembolism

ABBREVIATIONS

VEGF	vascular endothelial growth factor
tPA	tissue type plasminogen activator
PAI-1	plasminogen activator inhibitor 1
lp(a)	lipoprotein(a)
DVT	deep vein thrombosis
a.u.	arbitrary units
PBS	phosphate buffered saline
GFAP	glial fibrillary acidic protein
RTQ-PCR	real time quantitative polymerase chain reaction
GAPD	glyceraldehydes-3-phosphate dehydrogenase

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ABSTRACT

Malignant gliomas are associated with risk of thromboembolism, but the molecular link between tumor and peripheral pro-coagulant status has not been elucidated. Vascular Endothelial Growth Factor (VEGF), tissue-type Plasminogen Activator (tPA), Plasminogen Activator Inhibitor-1 (PAI-1) and lipoprotein (lp) (a) influence the pro-coagulant status. To assess whether the presence of the tumor influenced the peripheral levels of VEGF, tPA, PAI-1 and lp(a), we studied the expression and secretion of VEGF, tPA, PAI-1 and lp(a) in glioma specimens, in peripheral blood and in primary glioma-derived cultures. We also measured lp(a), VEGF, tPA and PAI-1 in the peripheral circulation of patients, before and after surgery for glioma.

VEGF, tPA and PAI-1 were expressed in glioma specimens. Glioma cells were indeed a major source of tPA and PAI-1; these molecules were significantly more expressed in glioma than in patient's blood cells. Lp(a) was rarely expressed in glioma specimens and not expressed in blood cells. In glioma, VEGF, tPA and PAI-1 were localized mainly in tumor cells; tPA was localized also in the extracellular matrix and PAI-1 in tumor vascular lumen. Glioma cells were indeed able to produce and release VEGF, tPA and PAI-1. After surgery, peripheral levels of VEGF and PAI-1 were increased, while tPA and lp(a) were unchanged.

The great amount of VEGF, tPA and PAI-1 produced by glioma could influence peripheral levels of these molecules. The partial resection of the tumor by surgery was not able to decrease plasma levels of these molecules.

INTRODUCTION

The association between cancer and deep vein thrombosis (DVT) has been described since 1865 by Prof. Trousseau and well documented over more than a century. Cancer itself is a risk factor for DVT, which occurs, in some cases, as a symptom of occult malignancy,[1] independently from surgery[2] and from adequate prophylaxis against post-operative DVT.[3] Several types of cancer are associated with increased risk of DVT, particularly high grade glioma.[4,5] In a wide group of patients that underwent surgery for high grade gliomas, we previously found high plasma levels of d-dimer (product of fibrinolysis), lipoprotein (lp) (a), homocysteine, Vascular Endothelial Growth Factor (VEGF), tissue-type Plasminogen Activator (tPA) and Plasminogen Activator Inhibitor-1 (PAI-1).[6] All these molecules could contribute to the coagulation cascade influencing several reactions and cross-interacting. Peripheral levels of homocysteine, VEGF, tPA, PAI-1 and lp(a) were high, in our glioma patients, independently from their genetic background.[6] It can be supposed that, in these patients, the peripheral pro-coagulant status may be influenced by the presence of the tumor. This hypothesis is supported by the observation that glioma cells produce Tissue Factor (TF),[7] which initiates the coagulation cascade. TF is upregulated by VEGF produced by endothelial cells of glioma environment.[8] PAI-1 could increase VEGF expression in glioma cell line,[9] inducing endothelial cell proliferation and, possibly, modifying pro-coagulant status in the tumor microenvironment. The correlation between the presence of intraluminal thrombosis in brain tumor vessels at surgery and the development of peripheral DVT in patients with glioma has been previously reported, further suggesting that intra-tumor pro-coagulant status could influence peripheral predisposition to DVT,[10] but the molecules possibly involved in these processes have not been described. Thus, the possibility that glioma cells produce and release lp(a), VEGF, tPA and PAI-1 in the tumor microenvironment and, from here, in the circulation, influencing the peripheral pro-coagulant status of patients with glioma, remains to be fully clarified and cellular source of VEGF, tPA and PAI-1 has not been fully investigated. Previous studies, in fact, documented the presence of these molecules in glioma specimens, but only in the form of

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mature protein. Because VEGF, tPA, PAI-1 and lp(a) are secreted proteins, their association in the tumor microenvironment could be due to peripheral recruitment or to local production. In the present work we studied the capacity of glioma cell to produce these procoagulant moleculs by analyzing mRNA expression of VEGF, tPA, PAI-1 and lp(a) in tumor specimens and, as comparison, in peripheral blood cells of patients with high grade glioma. We then analyzed the localization of mature proteins in tumor sections. We also evaluated VEGF, tPA and PAI-1 mRNA expression and protein secretion by glioma cells isolated from tumor specimens and briefly cultured in vitro. The role played by the tumor in the modification of peripheral procoagulant status has been investigated by measuring of plasma levels of VEGF, tPA, PAI-1 and lp(a) before and after surgery.

MATERIALS AND METHODS

Patients. Patients (n = 31, 22 male and 9 female, mean age 52.6 years, SD 10.4) were enrolled over a period of one year. At surgery, we received peripheral blood; the tumor was used mainly for pathological diagnosis and, when specimens were large enough, the remaining part of the tumor was used for this study. Patients gave informed consent to donate anonymous biological samples for research use. The histological diagnoses of tumor, performed by two independent pathologists, on these 31 samples were: glioblastoma multiforme (WHO grade IV, n = 19), anaplastic astrocytoma (WHO grade III, n = 6), anaplastic oligoastrocytoma (WHO grade III, n = 4), anaplastic oligodendroglioma (WHO grade III, n = 2). We also obtained a surgical specimen from an epileptic patient with medial temporal sclerosis, that we tested as non-tumor control in immunoistochemical experiments.

The amount of tumor resection was evaluated comparing the MR images of the tumors before surgery with MR images perfomed within 48h post-surgery: in all patients of this study, a residual tumor mass was detectable, consistent with a partial resection (67% ± 15%) of a large tumor mass (52 ± 24 mm).

In this cohort, one patient incurred in a DVT and died after a few weeks; 6 other patients died during the follow-up due to disease progression. After surgery, patients with glioma received radiotherapy (external bean radiotherapy 60 Gy) and were treated with 1,3-bis(2-chloroethyl)-1-nitrosourea and cisplatinum every six weeks, with the first cycle delivered prior to radiotherapy.

Healthy control group (n = 14) includes healthy blood donors and healthy volunteer staff personnel of National Neurological Institute C. Besta. Healthy control samples were taken during blood donation or during routinely control of health status of the staff of our Institute.

A small group of patients (n = 4) having non-tumor neurological diseases (one cluster headache, two neurovascular conflicts and one Parkinson) underwent brain surgery and participated to this study with blood donation before and 6–15 days after surgery. These samples were used to measure VEGF, tPA and PAI-1 plasma levels as controls in non-glioma brain surgery patients.

Patients specimens. Tumor samples for research use were stored at 4°C in Dulbecco's Modified Eagle Medium (DMEM, GibcoInvitrogen, Paisley, UK) containing penicillin 100 U/ml (Gibco), streptomycin 50 ng/ml (Gibco) and L-glutamine 200 mM (Sigma, St. Louis, MO), hereafter referred to as complete medium, added with 10% Fetal Bovine Serum (FBS, Gibco). The size of the tumor samples available for this study was variable, so that tissues were divided for total RNA extraction (n = 31), paraformaldehyde inclusion (n = 22), and, in the only ten cases in which we received tumor samples large enough, also for primary cell culture.

Peripheral blood samples were taken, by venipuncture in vacutainer tubes containing Na-Citrate or EDTA as anticoagulant, immediately before surgery (n = 31) and at two following time points: 6–30 days (n = 18) and 31–120 days (n = 21). During the follow up, we did not obtain blood samples from all patients (some of the patients living outside the region did not undergo blood sampling at both time points during follow-up).

Tumor cell primary cultures. Tumor samples of ten patients were mechanically minced with scissors in cell culture Petri dishes (Corning, Cambridge, MA) and incubated in 20% FBS complete medium at 37°C and 5% CO_2. At confluence, cells were detached with trypsin (Gibco), washed with phosphate buffered saline (PBS, Gibco) and equally distributed in 6 wells plate (Corning) with 1 ml of 10% FBS complete medium. Semiconfluent cultures (about 300,000 cells/well) were then harvested: medium was replaced and cells were incubated for 3 or 24 h. After these times, supernatants of two wells, for each time point, were collected and stored at -20°C until used. Adherent cells of two wells for each time point were treated with trypsin, washed with PBS and lysed with the RNeasy lysis buffer (Qiagen, Hilden, Germany) for RNA extraction; one well was treated for cytofluorimetric analyses of Glial Fibrillary Acidic Protein (GFAP) expression, to evaluate the percentage of GFAP positive glial cells in our primary cultures. Briefly, cells were incubated with a rabbit polyclonal anti-GFAP (Dakocytomation, Carpinteria, CA) at 4°C for 30'. After 1 wash with PBS, cells were incubated at 4°C for 30' with anti-rabbit secondary antibody conjugated with tetramethylrhodamine (TRITC, Dakocytomation). PBS washed cells were analyzed by cytofluorimetry (FACSTAR PLUS Cytofluorimeter, Beckton Dickinson, Heidelberg, Germany).

Real time quantitative-polymerase chain reaction (RTQ-PCR). Total RNA was extracted using commercial kits (for cells and tissues: RNeasy Mini Kit, Qiagen; for blood: RNA fast, Molecular System, San Diego, CA). We obtained cDNA using 10 U M-MLV reverse transcriptase (Promega, Madison, WI), 0.2 U RNase inhibitor (Promega), 50 μM random hexamers (Amersham Biosciences, Piscataway, NJ), 0.5 mM each dNTP (Amersham Biosciences, Piscataway, NJ). For each amplification, 30 ng cDNA were used with an appropriate amount of RTQ-PCR Universal Master Mix (Applied Biosystems/Roche, Branchburg, NJ) and primers and probe for glyceraldehydes-3-phosphate dehydrogenase (GAPD), VEGF, tPA, PAI-1 and lp(a) (Gene Assay on demand, Applied Biosystems/Roche). All primers and probe sets were controlled for sequence accuracy and, as positive control, functionally tested with a pooled human cDNA (Applied Biosystems/Roche). Amplification was run in duplicate with negative controls (no cDNA) included. Acquisition of fluorescence emission at each cycle was performed in a 5700 Sequence Detection System Instrument (Applied Biosystems/Roche). In order to normalize all samples, the threshold cycle (the start of exponential amplification, Ct) of target gene of each sample was subtracted of the threshold cycle of its corresponding housekeeping gene GAPD, obtaining the ΔCt value: the higher ΔCt, the minor expression level. We consider expression levels as high, when ΔCt values go from 0 to 5, moderate from 5.01 to 10 and low from 10.01 to 25.

To evaluate whether isolated glioma cell expressed VEGF, tPA and PAI mRNAs, we performed RTQ-PCR also in mRNA from glioma cell primary cultures using specific primers/probe (Assay on demand, Applied Biosystem). In this case, the presence of VEGF, tPA and PAI-1 mRNA was not quantified but simply evaluated by electrophoresis run in 1% agarose gel stained with ethidium bromide amplification products obtained at end of the reactions.

Measurement of secreted protein. We measured tPA, PAI-1 and lp(a) in plasma obtained from Na-Citrate vacutainer or in cell culture supernatants and VEGF in plasma from EDTA vacutainer or in cell culture supernatants. Values were determined by commercial enzyme immunoassay kits (tPA, PAI-1 and lp(a): Hyphen BioMed, Andrésy, France; VEGF: Pierce Endogen, Rockford, USA), running each sample in duplicate. For ELISA of cell culture supernatants, standard samples were diluted in the same medium used for test samples. The value 0 pg or ng/ml was the undiluted 10% FBS medium and had absorbance typical of blank value.

Immunohistochemistry. Tissues were included in 4% paraformaldehyde for 24 h and then paraffin embedded. Sections of 3 μm were mounted on glasses. After de-paraffinization procedure, sections were incubated with 3% H_2O_2 for 15' to block endogenous peroxidase activity and then incubated with Protein Block Serum-Free (Dakocytomation) for 30'. Glasses were incubated overnight with monoclonal mouse anti-human VEGF antibody (R&D System, Minneapolis, MN) diluted 1:100, polyclonal rabbit anti-human PAI-1 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100, polyclonal rabbit anti-human tPA (Santa Cruz Biotechnology) diluted 1:50,

Table 1 **ΔCt values indicating the mRNA expression in samples of patients with high grade glioma: the lower ΔCt, the higher expression**

	Tumor	Tumor vs Patients Blood: p Values	Patients Blood	Healthy Controls Blood
VEGF	4.13 ± 2.16	p not significant	4.26 ± 3.03	5.35 ± 1.74
tPA	5.23 ± 1.29	p < 0.0001	10.06 ± 3.70	12.10 ± 2.20
PAI-1	5.57 ± 2.86	p = 0.0126	7.37 ± 2.22	8.34 ± 1.75
Lp(a)	13.55 ± 5.51		undetectable	undetectable

and then with monoclonal anti-mouse or anti-Rabbit peroxidase conjugated (Dakocytomation) for 1h. Visualization was performed using di-aminobenzidine (DAB, Dakocytomation) as substrate. Finally, the sections were counterstained with hematoxylin. Negative controls (i.e., sections in which the primary antibody was substituted by non-immune serum) were also stained each run.

Staining was evaluated by two observers blinded to diagnosis and sections were classified as positive (positive cells >10%) or negative (considering separately tumor and endothelial cells). Results were expressed as percentage of positive samples out of the total number of investigated patients. For each sample, the two pathologists indicated also the cell type where the immuno-staining was localized.

Statistical analyses. The values of ΔCt resulting from amplifications of cDNA from tumor and from blood cells have been compared using Mann Whitney test for non-parametric data.

Linear regression analyses were used to evaluate the relation between tumor size before surgery and plasma levels of VEGF, tPA and PAI-1 before surgery.

Pre and after- surgery plasma levels of VEGF, tPA, PAI-1 and lp(a) were compared with Wilcoxon test for non-parametric paired data: for each patient, the post-surgery value has been compared with the corresponding presurgery value.

RESULTS

Expression of VEGF, lp(a), tPA and PAI-1 mRNA in tumor specimens, in peripheral blood and in glioma cell cultures. Expression of mRNAs (Table 1) was quantified by RTQ-PCR in 31 tumor specimens and in blood samples of 18 patients with high grade glioma and in blood samples of 14 healthy controls. We observed that VEGF was expressed at high levels in high grade glioma samples as well as in peripheral blood cells of patients before surgery. Moderate amount of VEGF was expressed in peripheral blood cells of healthy controls, not differently from blood cells of glioma patients. The expression of tPA was significantly higher in tumors compared to blood cells of patients ($p < 0.0001$), that expressed low levels of tPA. The amount of tPA expressed in blood cells of healthy controls was low, not statistically different from that observed in blood cells of glioma patients. The expression of PAI-1 mRNA in high grade tumor samples was significantly higher than that observed in blood cells ($p = 0.0126$) of patients with glioma. The amount of PAI-1 expressed in blood cells of healthy donor was similar to that observed in blood of patients with glioma. The expression of lp(a) mRNA was detectable, at low levels, only in 30% of tumor samples analyzed while it was undetectable in blood cells (Table 1).

Tumor specimens are composed by several cell types. To study the production of VEGF, tPA, PAI-1 and lp(a) in isolated glioma cells, we measured mRNA expression in ten glioma primary cultures. In vitro cultured cells appeared morphologically as glioma cells and expressed GFAP at variable levels (18–90% positive cells), as seen by cytofluorimeter analysis (not shown). GFAP variability of expression depends on tumor grade: the higher the tumor grade, the lower the GFAP expression.[11] VEGF, tPA, PAI and lp(a) amplification products were run by electrophoresis in ethidium bromide stained gel. All tumor cells cultures expressed VEGF, tPA and PAI-1 mRNAs and did not express lp(a). Figure 1 shows RTQ-PCR end-point amplification products of five representative cell lines [lp(a) not shown].

Expression and secretion of VEGF, tPA and PAI-1 proteins. VEGF, tPA and PAI-1 protein expression was studied in tumor sections and in one case of brain tissue from an epileptic patient (medial temporal sclerosis) by immunohistochemistry, while the secreted forms of these proteins were evaluated in peripheral circulation and in glioma cell culture supernatants by ELISA.

We analyzed the presence of VEGF, tPA and PAI-1 in 22 tumor specimens. We found the presence of VEGF in tumor cells in all cases and in tumor vessels endothelial cells in 50% of cases (Fig. 2A), while we did not observe the presence of VEGF in tumor border and in non-neoplastic cells of these sections. VEGF was expressed also in some endothelial cells of epileptic brain tissue, but not in other cell types of this sample (not shown). We then observed that tPA was present in tumor cells of 65% of cases of glioma. In 32% of cases, tPA was also detected in the extracellular matrix (Fig. 2B), while it was undetectable in endothelial cells and in non-neoplastic cells of all sections. No signal of tPA expression was detected in the brain tissue of the epileptic patient. PAI-1 was strongly expressed in tumor cells of all cases (Fig. 2C) and in endothelial cells of 70% of cases (Fig. 2D); in some cases, PAI-1 was present also in the lumen of tumor vessels. We did not find PAI-1 expression in non-neoplastic cells of tumor sections and neither in the brain epileptic tissue.

Immunohistochemical data suggested that a major source of VEGF, tPA and PAI-1 in brain of patients with glioma were tumor cells. We cultured in vitro glioma cells from ten patients for 3 h and 24 h and we then harvested the supernanants. VEGF, tPA and PAI-1 ELISA kits required a different quantity of supernatant to be tested: the following data are referred to the indicated number of cells corresponding to the amount of supernatants tested. In supernatant of about 15,000 cells, we found 541.6 pg/ml (SD 1059.4) of VEGF secreted after 3 h and 527.6 pg/ml (SD 586.2) after 24 h (Fig. 3A). The secretion of tPA by 300,000 glioma cell culture supernatants was 0.49 ng/ml (SD 0.31) after 3 h and 0.80 ng/ml (SD 0.59) after 24 h (Fig. 3B). PAI-1 protein secreted from 7,500 glioma cells after 3 h was 11.94 ng/ml (SD 4.2) and, after 24 h, 19.71 ng/ml (SD 1.23) (Fig. 3C).

Post surgical follow up of plasma levels of proteins involved in VTE. We measured the concentration of VEGF, tPA, PAI and lp(a) in the peripheral circulation of 31 patients with glioma, before surgery. At this starting time point, the tumors were measured on magnetic resonance imaging. Peripheral levels of PAI-1 significantly correlate with the tumor size (linear regression analysis: p value 0.0466), while VEGF and tPA plasma values did not significantly correlate with tumor size (p values 0.62 and 0.97, respectively). The

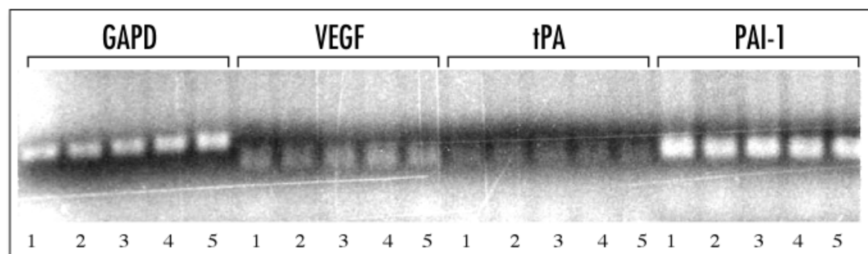

Figure 1. Expression of GAPD, VEGF, tPA and PAI-1 in glioma primary cultures. End point RTQ-PCR visualized by ethidium bromide stained agarose gel showed that all cell cultures (in the figure are shown 5 representative cell cultures out of ten analysed) expressed VEGF, tPA and PAI-1 (as well as housekeeping GAPD) mRNAs.

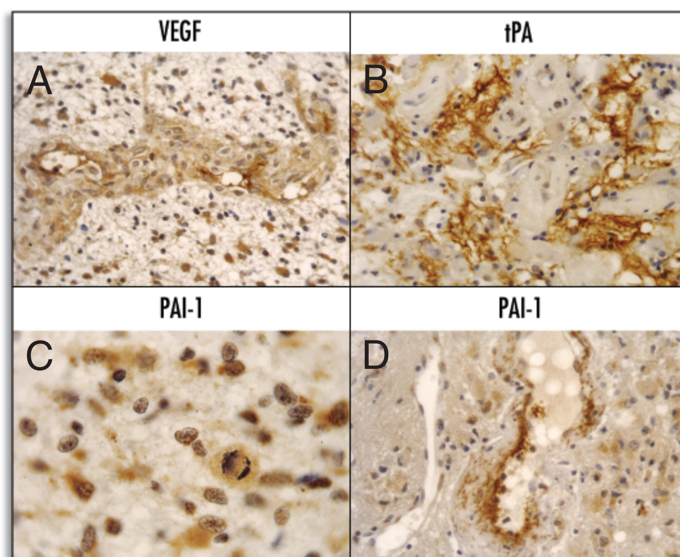

Figure 2. Immunohistochemical analyses of VEGF, tPA and PAI-1 expression in tumor specimens. (A) VEGF labelling in some tumor and endothelial cells of glioma tissue. (B) tPA immunostaining localized in some glioma cells and in the extracellular matrix. (C) PAI-1 signal in cytoplasm of glioma cells. (D) An example of two vessels of glioma specimen, one negative and one positive for PAI-1 immunostaining.

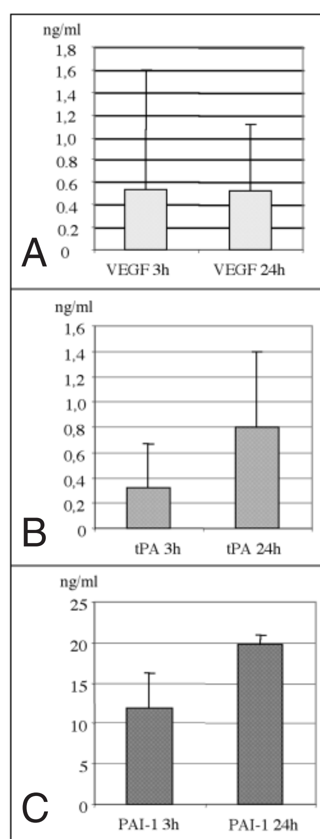

Figure 3. Measure of secreted VEGF, tPA and PAI-1 in glioma cell culture supernatants. (A) VEGF secreted after 3 h (mean 541.6 pg/ml, range 8.8–3151.2 pg/ml, SD 1059.4) and after 24h (mean 527.6 pg/ml, SD 586.2, range 63.9–1726.6). (B) tPA secreted after 3 h (mean 490 pg/ml, SD 310, range 80–900) and after 24 h (800 pg/ml, SD 590, range 120–2040). (C) PAI-1 secreted after 3 h (11.94 ng/ml, SD 4.20, range 8.1–19.6) and after 24 h (19.71, SD 1.23, range 18.8–21.6).

mean amount of tumor resection was 67 ± 15%, as measured with magnetic resonance. We then evaluated variation of VEGF, tPA and PAI-1 plasma values after surgery: inter-individual normal variability of molecules plasma levels suggests to express data as percentage of variation, rather than concentration. VEGF increased significantly 6–30 days after surgery (variation: + 156.8%, p = 0.025). At the following time point, VEGF plasma levels were similar to those measured before surgery (variation: -0.3%, p = 0.43) (Fig. 4A). Variation of tPA after surgery was not significant, nor after 6–30 days (+27.6%, p = 0.19), neither after 30–120 days (+17.4%, p = 0.36) (Fig. 4B). After surgery, plasma levels of PAI-1 increased significantly after 6–30 days (+90.6%, p = 0.005), as well as after 31–120 days (+59.5%, p = 0.023) (Fig. 4C). Lp(a) post-surgery plasma levels were not significantly different compared to those measured before surgery (after 6–30 days variation was +37.4%, p = 0.0995; after 31–120 days variation was +62.4%, p = 0.33) (Fig. 4D).

The increase of VEGF, tPA and PAI-1 during the first month after surgery could be attributable to surgery bleeding, independently from the tumor. To confirm this observation we measured the variations of VEGF, tPA and PAI-1 plasma levels also in a small group of patients before and 6-15 days after neurosurgery for other than tumor diseases. We observed an increase of VEGF (mean 276 ± 59.2%), tPA (mean 43.5 ± 94.5%) and PAI-1 (mean 52.99 ± 171.14%) plasma values. These increases were not statistically significant, due to the low number of patients in this group, but confirm that neurosurgery per se induce a temporary increse of pro-coagulant status. It is notable that, in patients with glioma, we measured increased levels of procoagulant molecules, expecially PAI-1, even long time after surgery.

DISCUSSION

DVT represents one of the main complications in patients with high grade glioma, not only during the peri-operative period, but also long after surgery.[2,4,5] Predisposition to DVT could be attributable to factors not genetically determined, including not only age,[12] tumor grade[4] and volume,[13] limb paresis, chemotherapy,[14] but also plasma pro-thrombotic status.[6] To identify plasma markers predicting DVT event in patients with glioma, the assessment of production and secretion in peripheral circulation of the candidate molecules by glioma cells is a prerequisite. We tested, as candidate molecules, VEGF, tPA, PAI-1 and lp(a). We observed that lp(a) was not expressed in glioma specimens and in peripheral blood cells of patients. The post-surgical levels of lp(a) were increased, but not significantly until 120 days of follow up. We observed high inter-individual variability, resulting in high standard deviation from the mean and in not significant p value. The increase of plasma lp(a) should therefore be attributable to hepatic source of this molecule, but the regulatory mechanism acting on lp(a) remains to be defined.

The expression of VEGF, tPA and PAI-1 has been previously studied in stabilized cell lines or in tumor tissues by immunohistochemistry. Cell lines represent a good experimental tool to study tumor cells, but the profound genetic modifications of cell lines may render them heterogeneous and not representative of the in vivo conditions: gene expression in these cells could be altered by their permanent in vitro life. Contrasting literature data, concerning the presence and the localization of VEGF, tPA and PAI-1 in tumor specimens,[15-24] prompted us to further define the capacity of glioma cells to produce and also to secrete VEGF, tPA and PAI-1. These are secreted proteins and thus their localization in a cell type could be due to receptor capture. For this reason we evaluated the production of VEGF, tPA and PAI-1 in both tumor specimens and in glioma cell isolated in cultures analyzing both mRNA and mature protein expression. We observed that glioma tumor cells, rather than endothelial cells of tumor vessels or circulating blood cells, could be indeed a major source of these molecules. Primary cell cultures demonstrated the capacity of glioma cells to produce and also secrete VEGF, tPA and PAI-1. Immunohistochemical images suggested also that tPA and PAI-1, localized in some cases in the extracellular matrix or in the vessel lumen, could be produced in the tumor environment and secreted in the circulation, thus possibly modifying the peripheral levels of these molecules. To test this hypothesis, we evaluated the modification of peripheral levels of VEGF, tPA and PAI-1 in a group of patients, after the resection of the tumor. We observed that plasma VEGF increased 6-30 days after surgery, but this could be a consequence of surgery bleeding. In fact, at longer

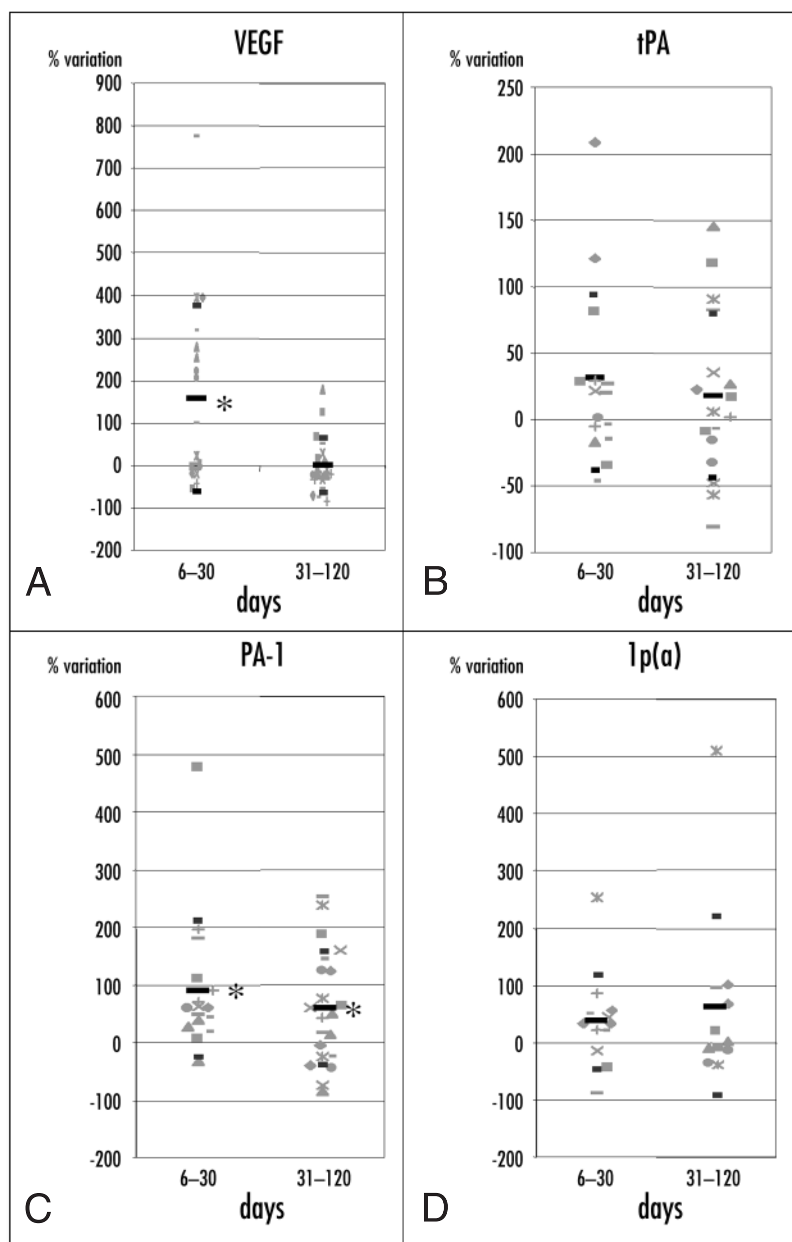

Figure 4. Variation of VEGF, tPA, PAI-1 and lp(a) after surgery. Data are expressed as percentage of variation with respect to values measured before surgery. Each individual is represented by a grey symbol. Mean values are represented by long black line and standard deviation are indicated with short black line. Star symbol (*) indicated significant variation ($p < 0.05$). VEGF percentages of variation were: +156.8% (SD 219.5, $p = 0.025$) 6–30 days after surgery; -0.3% (SD 65.4, $p = 0.43$) after 31–120 days (A). After surgery tPA variations were: +27.6% (SD 65.8, $p = 0.19$) at 6–30 days; +17.4% (SD 61.7, $p = 0.36$) at 31–120 days (B). After surgery, PAI-1 varied as follow: +90.6% (SD 118.2, $p = 0.005$) at 6–30 days; +59.5% (SD 98.4, $p = 0.023$) at 31–120 days (C). Lp(a) plasma post surgery variations were: -37.4% (SD 82.8, $p = 0.0995$) after 6–15, +62.4 (SD 156, $p = 0.33$) after 31–120 days (D).

time point (31–120 days), mean VEGF plasma value was not different compared to the presurgery mean value. Peripheral levels of tPA were also increased, but not significantly, while PAI-1 plasma levels were significantly increased for a long time after surgery. It is still possible that PAI-1 increase was due to surgery injury,[25] but it was increased even at 31-120 days after surgery, suggesting a correlation with the pathology of patients, rather than with surgery. On the other hand, the role of surgery per se on the short-term increase in plasma VEGF, tPA and PAI-1 was supported by our data on non-glioma patients undergoing brain surgery. We have to notice that, in the patients of this study, the resection of the tumor was always partial. The partial resection of a source of VEGF, tPA and PAI-1, did not decrease, but in some cases increased, the peripheral levels of these molecules. It has been reported that survival of patients with a partial resection is not different compared to that of patients not operated,[26] suggesting that, after a disease free time, the progression of operated tumors occurs more rapidly than in non-operated tumors. We point out that surgical resection of high grade glioma was not followed by a decrease, but rather by an increase of plasma pro-coagulant status due to molecules such as VEGF and PAI-1. These molecules are mainly involved in the progression of the tumor, influencing tumor neo-angiogenesis, extracellular matrix degradation and tumor cell spreading. Although our study was meant to demonstrate that these factors are produced and secreted by glioma cells, we reasoned that the progression of the tumor could have, as a consequence, the modification of pro-coagulant status, thus explaining the surgery-independent occurrence of DVT and the poor prognosis associated with high levels of these molecules reported in previous studies in the literature.[27,28] The utility of life long anticoagulant therapy and of antiangiogenic drugs in patients with high grade glioma is under evaluation in several clinical trials.[29,30] This study provides further evidence demonstrating that glioma cells produce molecules involved in coagulation and in tumor progression. Partial surgery did not modify plasma levels of these factors. Prospective studies in high grade glioma patients will allow to identify subpopulation at higher risk for DVT/PE.

References

1. Otten HM, Prins MH. Venous thromboembolism and occult malignancy. Thromb Res 2001; 102:V187-94.
2. Marras LC, Geerts WH, Perry JR. The risk of venous thromboembolism is increased throughout the course of malignant glioma: An evidence-based review. Cancer 2000; 89:640-6.
3. Flordal PA, Bergqvist D, Ljungstrom KG, Torngren S. Clinical relevance of the fibrinogen uptake test in patients undergoing elective general abdominal surgery—relation to major thromboembolism and mortality. Fragmin Multicentre Study Group. Thromb Res 1995; 80:491-7.
4. Brandes AA, Scelzi E, Salmistraro G, Ermani M, Carollo C, Berti F, et al. Incidence of risk of thromboembolism during treatment high-grade gliomas: A prospective study. Eur J Cancer 1997; 33:1592-6.
5. Silvani A, Salmaggi A, Eoli M, Lamperti E, Boiardi A. Venous thromboembolism in malignant glioma patients treated by chemoradiotherapy. Neurol Sci 2003; 24:272.
6. Sciacca FL, Ciusani E, Silvani A, Corsini E, Frigerio S, Pogliani S, et al. Genetic and plasma markers of venous thromboembolism in patients with high grade glioma. Clin Cancer Res 2004; 10:312-7.
7. Guan M, Jin J, Su B, Liu WW, Lu Y. Tissue factor expression and angiogenesis in human glioma. Clin Biochem 2002; 35:321-5.
8. Takano S, Tsuboi K, Tomono Y, Mitsui Y, Nose T. Tissue factor, osteopontin, alphavbeta3 integrin expression in microvasculature of gliomas associated with vascular endothelial growth factor expression. Br J Cancer 2000; 82:1967-73.

9. Hjortland GO, Lillehammer T, Somme S, Wang J, Halvorsen T, Juell S, Hirschberg H, Fodstad O, Engebraaten O. Plasminogen activator inhibitor-1 increases the expression of VEGF in human glioma cells. Exp Cell Res 2004; 294:130-9.
10. Rodas RA, Fenstermaker RA, McKeever PE, Blaivas M, Dickinson LD, Papadopoulos SM, et al. Correlation of intraluminal thrombosis in brain tumor vessels with postoperative thrombotic complications: A preliminary report. J Neurosurg 1998; 89:200-5.
11. Peraud A, Mondal S, Hawkins C, Mastronardi M, Bailey K, Rutka JT. Expression of fascin, an actin-bundling protein, in astrocytomas of varying grades. Brain Tumor Pathol 2003; 20:53-8.
12. Ruff RL, Posner JB. Incidence and treatment of peripheral venous thrombosis in patients with glioma. Ann Neurol 1983; 13:334-6.
13. Sawaya R, Zuccarello M, Elkalliny M, Nishiyama H. Postoperative venous thromboembolism and brain tumors: Part I. Clinical profile. J Neurooncol 1992; 14:119-25.
14. Dhami MS, Bona RD, Calogero JA, Hellman RM. Venous thromboembolism and high grade gliomas. Thromb Haemost 1993; 70:393-6.
15. Zhou YH, Tan F, Hess KR, Yung WK. The expression of *PAX6*, *PTEN*, vascular endothelial growth factor, and epidermal growth factor receptor in gliomas: Relationship to tumor grade and survival. Clin Cancer Res 2003; 9:3369-75.
16. Johansson M, Brannstrom T, Bergenheim AT, Henriksson R. Spatial expression of VEGF-A in human glioma. J Neurooncol 2002; 59:1-6.
17. Nagashima G, Suzuki R, Asai J, Fujimoto T. Immunohistochemical analysis of reactive astrocytes around glioblastoma: An immunohistochemical study of postmortem glioblastoma cases. Clin Neurol Neurosurg 2002; 104:125-31.
18. Sawaya R, Highsmith R. Hyalinizing plasmacytic granulomatosis of the falx. Am J Surg Pathol 1988; 12:308-13.
19. Sawaya R, Ramo OJ, Glas-Greenwalt P, Wu SZ. Plasma fibrinolytic profile in patients with brain tumors. Thromb Haemost 1991; 65:15-9.
20. Kinder DH, Berger MS, Mueller BA, Silber JR. Urokinase plasminogen activator is elevated in human astrocytic gliomas relative to normal adjacent brain. Oncol Res 1993; 5:409-14.
21. Landau BJ, Kwaan HC, Verrusio EN, Brem SS. Elevated levels of urokinase-type plasminogen activator and plasminogen activator inhibitor type-1 in malignant human brain tumors. Cancer Res 1994; 54:1105-8.
22. Bindal AK, Hammoud M, Shi WM, Wu SZ, Sawaya R, Rao JS. Prognostic significance of proteolytic enzymes in human brain tumors. J Neurooncol 1994; 22:101-10.
23. Kono S, Rao JS, Bruner JM, Sawaya R. Immunohistochemical localization of plasminogen activator inhibitor type 1 in human brain tumors. J Neuropathol Exp Neurol 1994; 53:256-62.
24. Arai Y, Kubota T, Nakagawa T, Kabuto M, Sato K, Kobayashi H. Production of urokinase-type plasminogen activator (*u-PA*) and plasminogen activator inhibitor-1 (*PAI-1*) in human brain tumours. Acta Neurochir (Wien) 1998; 140:377-86.
25. Dietzmann K, von Bossanyi P, Krause D, Wittig H, Mawrin C, Kirches E. Expression of the plasminogen activator system and the inhibitors PAI-1 and PAI-2 in posttraumatic lesions of the CNS and brain injuries following dramatic circulatory arrests: An immunohistochemical study. Pathol Res Pract 2000; 196:15-21.
26. Lacroix M, Abi-Said D, Fourney DR, Gokaslan ZL, Shi W, DeMonte F, et al. A multivariate analysis of 416 patients with glioblastoma multiforme: Prognosis, extent of resection, and survival. J Neurosurg 2001; 95:190-8.
27. DeClerck YA, Mercurio AM, Stack MS, Chapman HA, Zutter MM, Muschel RJ, et al. Proteases, extracellular matrix, and cancer: A workshop of the path B study section. Am J Pathol 2004; 164:1131-9.
28. Noel A, Maillard C, Rocks N, Jost M, Chabottaux V, Sounni NE, et al. Membrane associated proteases and their inhibitors in tumour angiogenesis. J Clin Pathol 2004; 57:577-84.
29. Goldhaber SZ, Dunn K, Gerhard-Herman M, Park JK, Black PM. Low rate of venous thromboembolism after craniotomy for brain tumor using multimodality profilaxis. Chest 2002; 122:1933-7.
30. Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat Rev Drug Discov 2004; 3:391-400.