VEGF and tPA Co-Expressed in Malignant Glioma

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Neovascularisation and migration of tumour cells are two features of highly malignant glioma. Vascular endothelial growth factor (VEGF) and tissue plasminogen activator (tPA) seem to be of importance in the process of malignancy. In the present study a topographical co-expression of tPA mRNA and VEGF mRNA ($VEGF_{121}$ and $VEGF_{165}$ isoforms) was demonstrated in the tumour edge of a rat malignant glioma, using in situ hybridisation. No signs of co-expression was seen in the normal brain tissue. In the normal brain the forms of VEGF mainly expressed were $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$. Further studies are required to show whether VEGF and tPA are produced by the same tumour cells and to elucidate the role of this co-expression.

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Important features of malignant glioma are a diffuse infiltrative growth and a high vascular density, which correlate with bad prognosis (1). The molecular basis of angiogenesis is far from completely understood, but the number of isolated factors involved in the angiogenic process are rapidly increasing (2). The endothelial cell mitogen vascular endothelial growth factor (VEGF), also described as vascular permeability factor (VPF), is one of the most potent and specific angiogenic factors known. Four different isoforms of VEGF has been described; $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, each differing with regard to the number of composing amino acids. They arise by alternative splicing. $VEGF_{121}$ and $VEGF_{165}$ are secreted forms, whereas $VEGF_{189}$ and $VEGF_{206}$ remain mainly cell associated (for review see (3)). VEGF is produced by glioma cells, especially under hypoxic conditions in vitro (4). It has also been isolated in human glioma tissue and in the cyst fluids of malignant tumours (5).

Both angiogenesis and migration of tumour cells are dependent on degradation of the extracellular matrix. Activation of the plasminogen activator-plasmin system seems to be of great importance in this degradation process. There are two plasminogen activators recognized, uPA and tPA. Several malignant tumours, including human malignant gliomas, have been shown to produce uPA (6, 7). tPA is a key enzyme in the fibrinolytic system which dissolves blood clots and maintains blood flow within the vasculature. The role and source of tPA during tumorigenesis is uncertain. tPA is produced by brain tumour cells (8–10), and is inversely correlated with dedifferentiation and invasiveness of human brain tumours in vivo (11, 12). Interestingly, VEGF increases the expression of the urokinase receptor in vascular endothelial cells and is also known to induce both tPA and uPA in a dose-dependent manner (13, 14). Based on this knowledge we have investigated the topographical distribution of tPA and VEGF mRNA in the intracerebral BT4C rat glioma model.

MATERIAL AND METHODS

Rat glioma model. The rat glioma cell line BT4C was used for intracerebral transplantation into inbred BD IX rats (for details see (15)). The BT4C cell line (a gift from Prof. Bjerkvig, Bergen, Norway), is a transplacental nitrosurea induced tumour which has been characterized as a gliosarcoma (15). The study was approved by the local animal ethics committee. Cells growing in log phase diluted in DMEM supplied with 5% BD IX rat serum to a concentration of 20 000 cells in 5 μl were implanted 3.5 mm to the right of bregma at a depth of 4.5 mm into the right caudate nucleus under stereotactic conditions. The insertion hole through the skull bone, was covered with bone wax. During the implantation procedure the cells viability was controlled by intermittent tryphan blue staining. A total of 6 animals were used. The animals were sacrificed 24 days after tumour implantation.

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RNA preparation. Total RNA was isolated from the BT4C tumours using TRIzolTM reagent (GIBCO BRL/ Life Technologies AB, Sweden) according to the manufacturers instructions.

Reverse transcriptase PCR. Reverse transcriptase PCR (RT-PCR) was performed on 1 μg total RNA from BT4C tumours using one Strand cDNA Synthesis kit (Boehringer Mannheim, Germany) and reverse primers for rat-tPA and rat-VEGF respectively (Table). cDNA synthesis was followed by a standard PCR protocol with denaturation at 98°C for 10 min, hot start at 80°C, 34 cycles at 94°C for 30 s, 45°C 30 s, and 72°C 1 min and, finally, 72°C for 5 min. Forward and reverse primers designed to amplify tPA and VEGF cDNA respectively (Table) was used for the RT-PCR reactions. We also used sequence specific primers to investigate which isoforms of VEGF that the tumour cells used in our tumour model expressed.

In situ hybridisation. Tumour bearing inbred BD-IX rats were rinsed from blood with 150 ml 150 mM NaCl and fixed by vascular perfusion in 4% paraformaldehyd/77mM Na_2HPO_4, 23 mM NaH_2PO_4 (PFA). The brains were dissected and further fixed in PFA at 4°C for 12 h, followed by cryoprotection in 30% sucrose/77 mM Na_2HPO_4, 23 mM NaH_2PO_4 for 24–48 h. The brains were then frozen in Tissue Tek (Miles Inc., USA) in a quick freeze station in cryostat CM 3000 (Leica, Germany). Sections were cryocut at a thickness of 10 mm and mounted on Super Frost/Plus microscope slides (Histolab Products, Sweden).

DIG labelled RNA-probe. The DIG-labelled anti rat-tPA probe was obtained using a DIG-RNA kit (Boehringer Mannheim) and T7 RNA polymeras on a plasmid-carrying a 412 base pair fragment from the 5′ end of the coding sequence of rat-tPA cDNA, previously linerized with Hind-III (Boehringer Mannheim). The orientation of the fragment was controlled by sequencing. The anti rat-VEGF probe was made as described above but synthesized from a 404 base pair fragment of the rat-VEGF coding sequence. Here the orientation was confirmed by restriction site analysis. Purification of the probe was made with NucTrap Probe Purification Columns (Stratagene).

DIG-label in situ hybridisation. The slides were fixed in 4% paraformaldehyd/77 mM Na_2HPO_4, 23 mM NaH_2PO_4, 0.15 M NaCl (PBS) for 10 minutes and then washed in PBS 3 × 3 min. Acetylation for 10 minutes in 1.33% v/v triethanolamine, 0.175% v/v HCL, 0.25% v/v acetic anhydride was performed to facilitate probe penetration. The slides were rinsed in PBS 3 × 5 minutes and then prehybridised in 50% formamide, 5 × SSC, 5 × Denhardt, 250 μg/ml Bakers yeast RNA and 500 mg/ml Herring sperm DNA in a chamber humidified with 50% formamide and 5 × SSC overnight in room temperature. Hybridization with DIG-labelled probes (50–200 ng/100 μl) was performed in 70°C overnight. The slides were protected from drying with siliconized coverslips which were removed after hybridization by dipping the slides into 5 × SSC 70°C, followed by posthybridization for one hour in 0.2 × SSC 70°C. The slides were then equilibrated at room temperature in 0.2 × SSC and 0.1 M Tris pH 7.5, 0.15 M NaCl (B1) before blocking in 10% fetal calf serum/B1 for at least one hour. Incubation with anti DIG Ap antibody (Boehringer Mannheim), diluted 1:5000 in 1% fetal calf serum/B1 was performed overnight in 4°C. The sections were protected from drying by parafilm during development, carried out in 175 μg/ml 5-Bromo-4-Chloro-3-Inolyl-Phosphate (BCIP), 450 μg/ml 4-Nitro Blue Tetra-zoliumchloride (NBT), 0.24 μg/ml levamisol diluted in 0.1 M Tris pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$ in darkness 1–3 days. The slides were put in 1 × TE 30 min to stop development and mounted in gelatine glycerol medium.

Table

Primers used for RT-PCR of tPA and VEGF respectively

Primer	DNA sequence
tPA-F	5′-AAA GCT GAC ATG GGA ATA TTG-3′
tPA-R	5′-ATG TTG TCT TGG ATC CAG TTC-3′
VEGF-F	5′-GTG CAC TGG ACC CTG GCT TTA C-3′
VEGF-R	5′-AGT GAT TTT CTG GCT TTG TTC TAT-3′

(F) = forward (R) = reverse.

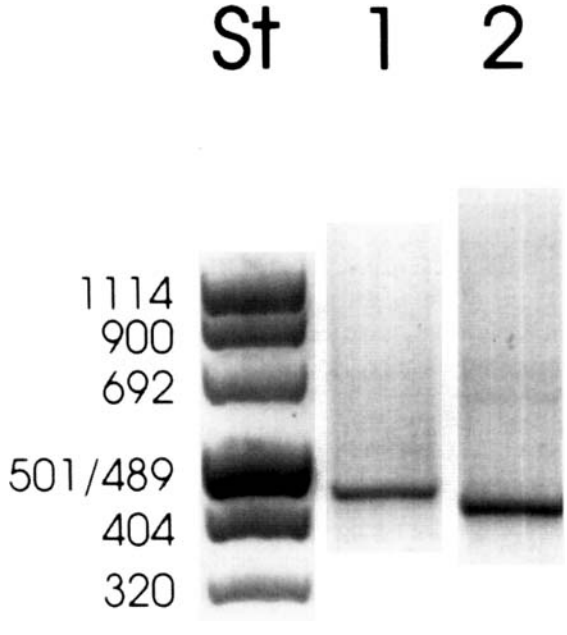

Fig. 1. RT-PCR on RNA from BT4C tumour. The amplification of a corresponding 412 bp tPA fragment is seen in lane 1, and a corresponding 404 bp VEGF fragment is seen in lane 2. Primers used for the PCR is listed in the Table. St = molecular weight standard.

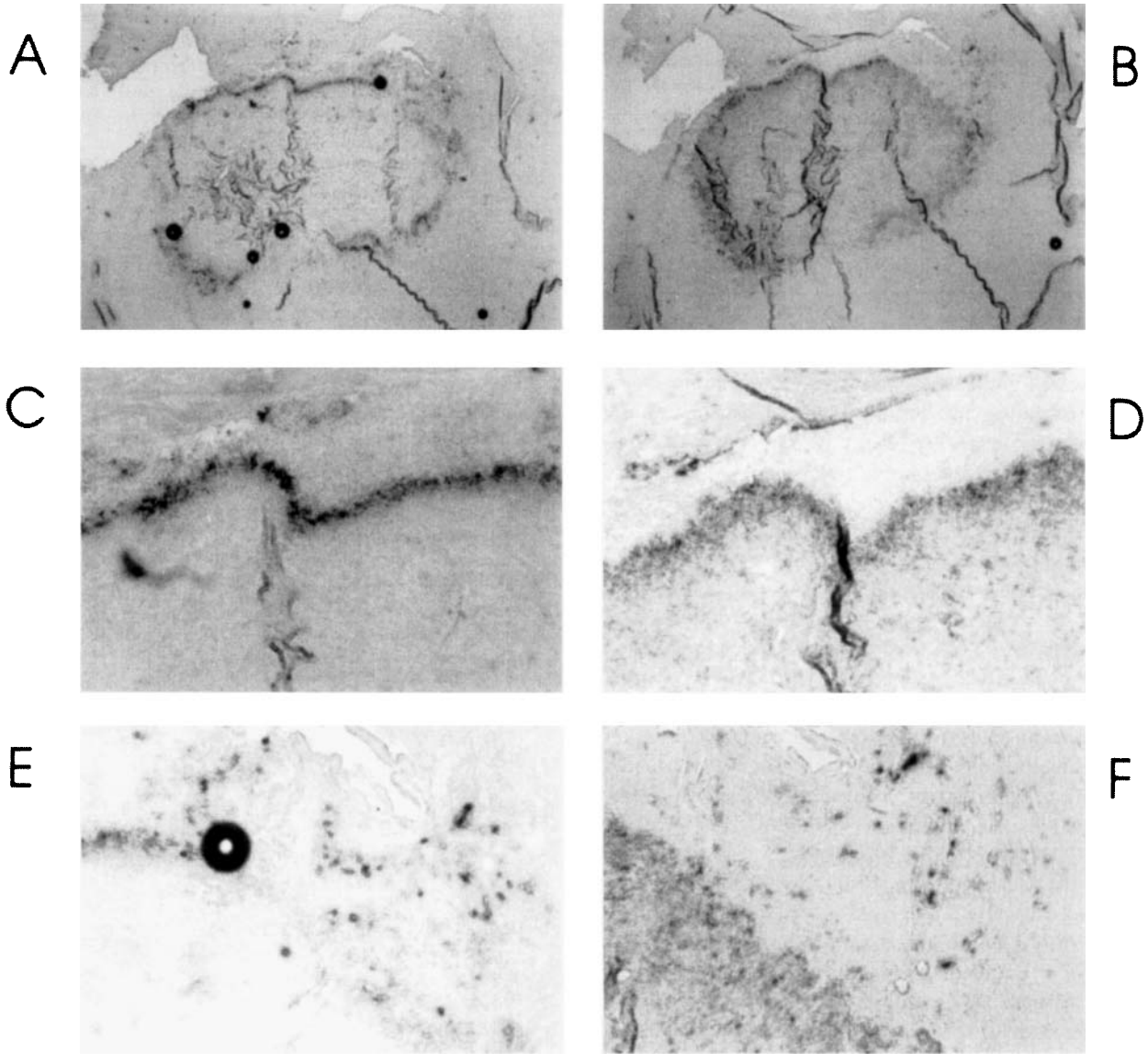

Fig. 2. In-situ hybridisation on the BT4C malignant glioma. A, C, and E show the expression of VEGF mRNA, B, D, and F the expression of tPA mRNA. A and B show an overview of the tumour, where the leading edge can be localised. C and D show a magnification of this edge. In E and F one can localise cells outside the tumour border which are positive for VEGF and tPA mRNA respectively.

RESULTS

The growth behaviour of the BT4C tumour was invasive with a clear tendency to perivascular growth with nests of tumour cells in the normal brain. Necrotic areas were not seen. The RT-PCR revealed the transcription of both tPA and VEGF mRNA in the malignant tumour (Fig. 1). RT-PCR on tumour tissue revealed the expression of the two isoforms $VEGF_{121}$ and $VEGF_{165}$ while in the normal brain $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ were the forms mainly expressed (data not shown).

The in situ hybridization demonstrated the presence of tPA mRNA throughout the tumour, with an obvious intensification in the tumour border adjacent to the healthy brain tissue. VEGF mRNA was exclusively localized to the invasive tumour edge. Few scattered cells outside the tumour border was positive for tPA mRNA and VEGF mRNA respectively (Fig. 2). In the normal brain, the ventricular ependyma was shown to be positive for tPA mRNA and plexus choroideus for VEGF.

DISCUSSION

The present study displays a co-expression of VEGF and tPA mRNA in a rat glioma model. The localization of both tPA and VEGF mRNA at the leading edge of the tumour is of certain interest. This phenomenon has earlier

been shown for uPA and its receptor (6, 16), but not for tPA or VEGF.

One can speculate whether the rapidly advancing and invasive tumour edge induces a transitional state of hypoxia and lack of nutrients during invasion and degradation of the normal brain which could explain the narrow upregulation of VEGF mRNA to meet the demand for neovascularization to improve oxygen and nutrients supply. The upregulation of VEGF will in turn induce production of plasminogen activators followed by degradation of extracellular matrix. This extracellular proteolysis is necessary for at least three elements of the angiogenic process; degradation of the basement membrane of the parent vessel, invasion of the interstitial extracellular matrix by migrating endothelial cells, and capillary lumen formation (for review see (17)). The breakdown of extracellular matrix promotes new capillaries to be formed and subsequently supply the hypoxic tumour edge with oxygen and nutrients. This creates an environment acceptable for the migrating cells which themselves have been equipped with surface-bound plasminogen activators to make migration possible. The importance of VEGF for tumorigenesis is supported by the fact that tumour progression in vivo is severely impaired by an antisense VEGF gene transfer approach to glioma cells (18).

The present study further suggests a plausible importance of interactions between growth factors and matrix degrading enzymes in the malignant process. More precisely, the spatial distribution of VEGF and tPA proposes that they could be co-regulated in the progression of malignant glioma. Whether these factors are produced by the same cells and work in concert are not fully clear, and the role of this co-expression requires further studies. The inhibition of C6 glioma growth by expression of antisense VEGF sequence (18) could be of interest in this context. The differences in expression of various subtypes of VEGF between tumour and normal brain tissue are also of interest to further elucidate.

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