American Journal of Pathology, Vol. 146, No. 5, May 1995

Up-Regulation of Urokinase and Urokinase Receptor Genes in Malignant Astrocytoma

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To understand the role of urokinase (u-PA) and the urokinase receptor (u-PAR) in malignant astrocytoma cell invasion of normal brain, astrocytic expression of u-PAR and u-PA mRNAs were analyzed by riboprobe* in situ *hybridization in astrocytoma and non-neoplastic brain biopsies. In eight of eight malignant astrocytomas (glioblastomas), u-PAR and u-PA mRNA expression was demonstrated, whereas in seven non-neoplastic brain biopsies, u-PAR and u-PA mRNAs were not expressed. In one of four low grade and all anaplastic astrocytomas u-PAR mRNA was expressed, although u-PA mRNA was undetectable. Consistent with the mRNA detection, u-PAR and u-PA proteins were expressed by malignant astrocytes in five of five glioblastoma biopsies. To study the tumor margin, U-251MG glioblastoma cells were propagated intracerebrally in a severe combined immunodeficient mouse xenograft (28 days), and u-PA mRNA was found to localize predominantly to the leading tumor edge, whereas u-PAR mRNA was expressed throughout the tumor. Furthermore, adherent human U-251MG glioblastoma cells* in vitro *expressed u-PAR and u-PA proteins, which localized to sites of integrin $\alpha v\beta 3$ cell-matrix contacts. These data indicate that co-expression of u-PAR and u-PA mRNAs and proteins marks the malignant astrocyte phenotype and that u-PA bound to u-PAR may play a role in glioblastoma cell invasion of normal brain by virtue of its expression at the leading tumor edge. (Am J Pathol 1995, 146:1150–1160)

Tumor cell protease digestion of the extracellular matrix has been shown to be a key step in tumor cell invasion.[1–4] Although it is likely that more than one class of protease is involved, ie, serine proteases and metalloproteases,[1–5] the serine protease urokinase (u-PA) has been shown to be expressed by several tumors *in vitro* and *in situ*.[1–3] Furthermore, u-PA production correlates with the invasive phenotype of breast, lung, and colon carcinoma and invasive melanoma.[1–3,6–13] u-PA proteolytically activates plasminogen to plasmin, and plasmin degrades a number of extracellular matrix proteins, including fibrin, fibronectin, laminin, brain myelin basic protein, and type IV collagen.[2,3,14–18] *In vitro* observations indicate that u-PA is bound to a cell membrane-specific receptor, the urokinase receptor (u-PAR), thereby localizing u-PA protease activity to specific sites on the cell membrane surface.[1–3,19–21] A major substrate for u-PA, plasminogen, also binds many cells through a specific receptor, and the cell surface activation of plasminogen to plasmin is enhanced when u-PA is bound to u-PAR.[1,3,20,22,23] Taken together, these observations suggest that u-PA bound to u-PAR is more efficient for promoting matrix protein degradation or pericellular proteolysis.[1,3,20,23] Additional evidence for the functional importance of u-PA binding to u-PAR in promoting tumor cell invasion is provided by studies demonstrating an inhibitory effect of anti-u-PAR antibodies on tumor metastasis *in vivo* and tumor cell migration/invasion *in vitro*.[24–27]

One tumor in which u-PA appears to be a functionally important serine protease, and thus in which it may play a role in the aggressive invasion of adjacent normal tissue, is malignant astrocytoma, also known as glioblastoma (GBM).[6,10,12,17,28] Several

Supported by National Institutes of Health National Cancer Institute Grant CA59958 (CLG), National Institute of Neurological and Communicative Disorders and Stroke Grant NS31096 (CLG and GYG), and a grant from the Parker B. Francis Foundation (MAO).

Accepted for publication January 23, 1995.

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After submission of this manuscript, Yamamoto et al (Cancer Research 1994, 43:5016–5020) reported increased expression of u-PAR mRNA in malignant gliomas as compared with normal brain *in vivo*.

previous studies have focused on the serine protease inhibitors that may block u-PA activity at the cell matrix interface.[10,12,29–32] Protease nexin I and a sodium dodecyl sulfate-activated thrombin-inhibiting serine protease inhibitor are thought to be major u-PA inhibitors in GBM tumor cells *in vivo*, although the type 1 plasminogen activator inhibitor is predominantly expressed in GBM blood vessels.[30,32] Expression of type 1 plasminogen activator inhibitor has previously been reported in platelets and endothelial cells.[33–37] Most recently, u-PA mRNA and protein have been described in malignant astrocytes within GBM tumors *in vivo*.[28] However, cell surface plasmin generation and therefore pericellular proteolysis are enhanced when u-PA is bound to u-PAR; thus, the importance of u-PA proteolytic action in these tumors is dependent upon both u-PAR and u-PA expression.[3] This concept is further supported by a recent study demonstrating that u-PA-mediated invasion of an extracellular matrix by GBM cells *in vitro* requires u-PAR expression.[26] To our knowledge there are no studies investigating u-PAR expression in astrocytic tumors *in vivo*. To determine the potential role of u-PAR in astrocytic tumors *in vivo*, we investigated the expression of u-PAR and u-PA mRNAs and proteins in various grades of astrocytic tumors. We found that 1), co-expression of u-PAR and u-PA mRNAs mark the malignant astrocyte phenotype; 2), u-PA co-localizes with u-PAR to the leading tumor edge in an intracerebral severe combined immunodeficient (SCID) mouse model of human GBM tumor, and 3), u-PA and u-PAR co-localize with integrin $\alpha v\beta 3$ to focal contacts in GBM cells *in vitro*. Taken together, these data indicate that u-PA bound to u-PAR probably plays a role in GBM cell invasion of normal brain.

Materials and Methods

Cells and Cell Culture

U-251MG human GBM cells[38] and HT1080 human fibrosarcoma cells[39] were maintained as previously described.[39,40] Both cell lines were routinely screened for mycoplasma bacterial contamination and were found to be negative.

Antibodies

Monoclonal antibody (MAb) anti-αv (LM142)[41] and MAb anti-β3 (AP3)[42] were previously described. Rabbit anti-human u-PA immunoglobulin (Ig)G (389), rabbit anti-human u-PAR IgG (393R), and MAb anti-human tissue-type plasminogen activator (373) were purchased from American Diagnostica (Greenwich, CT). Rabbit anti-human fibronectin receptor (anti-$\alpha 5\beta 1$) (GIBCO BRL, Grand Island, NY) and MAb anti-vinculin (Sigma Chemical Co., St. Louis, MO) were also purchased. The MAb anti-human u-PAR (3936; American Diagnostica) was a kind gift from Dr. Francois Booyse (The University of Alabama at Birmingham). Goat anti-mouse IgG and goat anti-rabbit IgG horseradish peroxidase conjugates were purchased (Bio-Rad Laboratories, Richmond, CA). Goat anti-rabbit IgG fluorescein isothiocyanate and goat anti-mouse IgG rhodamine conjugates were purchased (Jackson Laboratories, West Grove, PA).

Tissue Collection

Paraffin blocks of various grade astrocytoma biopsies and resections as well as non-neoplastic, consecutive brain biopsies from untreated patients were collected from the tissue archives at the University of Alabama Hospital. These samples had been formalin fixed before paraffin embedding. To assure optimal mRNA preservation, paraffin blocks from consecutive cases in which the tissue was placed into formalin fixative in the operating room were further analyzed. Fresh astrocytoma biopsies and resections were frozen for immunohistochemistry as previously described.[40] All tissue collection was in accordance with the Human Subjects Committee at The University of Alabama at Birmingham. Astrocytic tumors were histologically graded according to the World Health Organization as follows: low grade astrocytoma, anaplastic astrocytoma, and high grade astrocytoma (GBM).[43]

Immunohistochemistry

Immunohistochemistry was performed by the indirect method on 3-μ cryostat sections after acetone fixation (10 minutes at −20 C) in the absence of serum, as described.[13] The secondary antibody was coupled to horseradish peroxidase and followed by 3,3′-diaminobenzidine substrate, as described.[40,44] Tissue sections were stained with 0.5% methyl green (Sigma Chemical Co.), dehydrated, coverslipped, and photographed under bright field microscopy. Rabbit anti-u-PA IgG, rabbit anti-u-PAR IgG, and MAb anti-u-PAR IgG reacted weakly with formalin-fixed and paraffin-embedded tissue sections.

cDNAs, Northern Blot Analysis, and Riboprobe Transcription

The following human cDNAs were gifts: a 1113-bp human u-PAR cDNA[23] in pBs and a 2200-bp human

u-PA cDNA[45–48] in pGEM 3Z from Dr. Francois Booyse. U-PA and u-PAR inserts were submitted to partial dideoxy sequencing (Sequenase Version 2.0 kit, United States Biochemical, Cleveland, OH) to verify the identity of the inserts. The u-PAR cDNA used for transcribing riboprobes was composed of the *Xba*I/*Eco*RI insert (bp 31 to 1144) of the published DNA sequence,[23] and the u-PA cDNA used for transcribing riboprobes was composed of the *Xba*I insert (bp 1 to 2200) of the published DNA sequence.[45–48] These DNA constructs were random primed with the Klenow fragment of DNA polymerase (Prime-a-Gene Kit, Promega, Madison, WI) and labeled with α-[^{32}P]-dCTP.[37] Total RNA was extracted from U-251MG and HT1080 cells with RNazol B (Tel-Test, Friendswood, TX) and hybridized with the u-PA and u-PAR DNA probes, as described.[37,49] Single bands of appropriate size verified the specific complementarity of cDNAs. Relative message size was determined with RNA markers (562 to 9488 bp RNA markers, Promega). Specific activity of the DNA probes was typically 1×10^8 cpm/μg. The 0.6-kb cyclophilin cDNA in pSP64 vector[50] was received from Dr. Etty Benveniste (The University of Alabama at Birmingham). A 562-bp human integrin αv subunit cDNA in pSP72 vector was previously described and used for sense riboprobe transcription after linearization with *Eco*RI.[49] U-PA and u-PAR constructs were linearized for antisense and sense riboprobe transcription.[37,49,51–53] Purified linearized DNA templates were used to transcribe antisense and sense transcripts with the SP6, T7, or T3 phage polymerase (Promega).[37,49,51–53] [^{33}P]UTP (DuPont, Boston, MA) was incorporated into the riboprobes for signal detection.[53] Specific activity of the riboprobes was typically 1×10^8 cpm/μg.

In Situ Hybridization Analysis

In situ hybridization was performed as previously described.[37,49,51–53] Formalin-fixed and paraffin-embedded tissue sections were deparaffinized in xylene (15 minutes at 37 C, three times). Subsequently, tissue sections were initially fixed in 4% paraformaldehyde (15 minutes), washed, treated with proteinase K (Sigma Chemical Co.) (1 μg/ml; 10 minutes at 22 C), washed, prehybridized (100 μl; 2 hours at 55 C), and hybridized overnight at 55 C (2.4×10^6 cpm/slide).[49,53] After hybridization, the tissue sections were processed with the stringency conditions previously described, exposed (1 to 3 weeks), and developed.[49,52,53] The hybridized sections were graded as positive if ≥4 silver grains were observed over a nucleus or perinuclear area in at least two exposures on two different hybridizations. The grading system was as follows: 4 to 6 grains, weakly positive (wk +); 7 to 12 grains, positive (+); and >12 grains, strongly positive (++), under ×40 magnification. Antisense cyclophilin riboprobe hybridized to approximately 95% of cells in tissue. For Table I, hybridization slides were graded as positive (wk +, +, or ++) if >1% of cells were positive (excluding cells in blood vessels). To determine the percentage of cells expressing a particular mRNA within tissue sections, positive cells in 20 random fields were counted under bright field microscopy at ×40 magnification.

U-251MG Intracerebral SCID Mouse Xenografts

C.B. 17 SCID mice were obtained from the SCID mouse colony operated by the Animal Resources Program at The University of Alabama at Birmingham under protocol 9401564. U-251MG cells were harvested with buffered EDTA, washed, and adjusted to 1.25×10^8 cells/ml in phosphate-buffered saline. With a 250-μl Hamilton syringe and a 30-gauge needle, 1.25×10^6 cells (10 μl) were injected intracerebrally with stereotactic guidance into the SCID mouse right basal ganglia.[54] Mice were monitored daily for neurological signs. At 28 days after injection, mice were euthanized and the brains immediately harvested and bisected coronally at the injection site and frozen.

Immunofluorescence

U-251MG cells were plated on coverslips previously coated with vitronectin (10 μg/ml in phosphate-buffered saline) and allowed to attach and spread for 16 hours in Aim V serum-free media (GIBCO BRL) with 1% bovine serum albumin.[55] The human vitronectin used for attachment was purified as described[56] and migrated on a reduced Coomassie blue-stained 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel as a 65/75-kd doublet, as described.[57] Coverslips were washed, and cells were fixed with 3% paraformaldehyde (15 minutes at 22 C) and then permeabilized with buffered 0.2% Triton X-100 (1 minute at 22 C).[55] For double antigen co-localization, coverslips were incubated with rabbit primary antibody (30 minutes at 22 C), washed, and incubated with mouse primary antibody (30 minutes at 22 C).[55] This was followed by incubation with goat anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (1:200; 30 minutes at 22 C) and goat anti-mouse rhodamine-conjugated secondary antibody (1:200; 30 minutes at 22 C) and mounting

upside down on glass slides, with storage at 4 C in the dark.[55] Immunofluorescent microscopy was performed on a Leitz DMRB microscope equipped for immunofluorescence and photomicrography.

Results

Expression of u-PAR and u-PA mRNAs in GBM Tumors

The specificity of the u-PA and u-PAR cDNAs in U-251MG GBM cells were validated by Northern blot analysis. A 2.3-kb u-PA message (Figure 1, lane 1) and a 1.5-kb u-PAR message (Figure 1, lane 3) were detected in U-251MG cells, as expected.[23,45–48] To determine whether u-PAR and u-PA mRNAs were expressed in various grades of astrocytic tumors and their localization, *in situ* hybridization was performed.[37,49,51–53] *In situ* hybridization of serial sections of eight human GBM biopsies demonstrated tumor cell expression of both u-PA and u-PAR mRNAs in cells from the same area of all eight tumors (Figure 2A, C, respectively). In four anaplastic astrocytoma tumors, all expressed u-PAR mRNA, but u-PA message was undetectable (Table I). In one of four low grade astrocytomas, only u-PAR mRNA was detected. In seven non-neoplastic brain biopsies, both u-PAR and u-PA messages were undetectable. The association between u-PAR mRNA expression and the histological grade (combining anaplastic astrocytomas and GBM tumors) is highly significant ($P = 0.00005$; χ^2). The association between u-PA mRNA expression and GBM tumors is also highly significant ($P < 0.00001$; Fisher exact test). Endothelial cells in all GBM tumors and in those anaplastic astrocytomas with endothelial cell hyperplasia expressed u-PAR and u-PA mRNAs (data not shown). Serial sections hybridized in parallel with sense integrin αv subunit, sense u-PA (Figure 2B), or sense u-PAR (Figure 2D) riboprobes were consistently negative in all tissues studied. These data clearly indicate that u-PAR and u-PA message levels correlate with the malignant astrocyte phenotype.

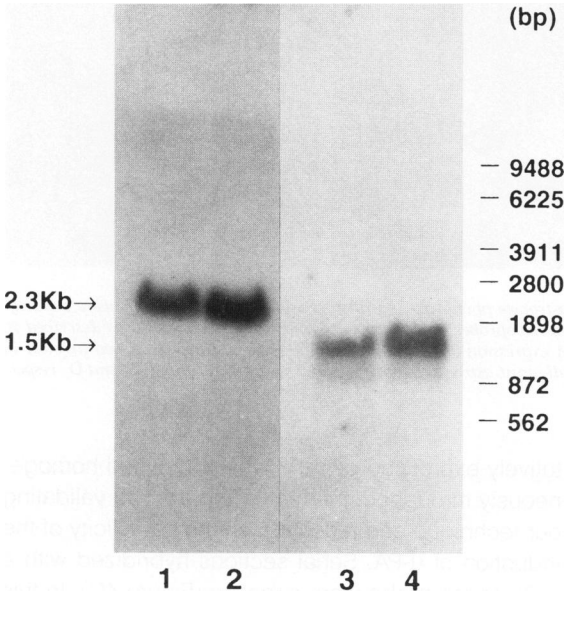

Figure 1. *Northern blot analysis of u-PA and u-PAR mRNAs in GBM cells. Total RNA was extracted from U-251MG GBM and HT1080 fibrosarcoma cells, and 30 μg/lane were electrophoresed on a urea/formaldehyde gel, transferred to nylon, and hybridized as described in Materials and Methods.*[37,49] *A 2.3-kb u-PA message is demonstrated in U-251MG cells* (lane 1) *and in HT1080 cells* (lane 2), *and a 1.5-kb u-PAR message is demonstrated in U-251MG cells* (lane 3) *and in HT1080 cells* (lane 4).

u-PAR and u-PA Protein Expression in GBM Tumors

To determine whether u-PA and u-PAR mRNAs were translated into protein, immunohistochemistry for u-PA and u-PAR proteins was performed on serial GBM acetone-fixed cryostat sections by the immunoperoxidase technique.[13] In five of five GBM tumors, u-PA and u-PAR proteins were detected in the malignant cells (Figure 3A, B, respectively), consistent with the *in situ* hybridization results. In serial sections of at least one GBM tumor, u-PA and u-PAR proteins clearly localized to the same malignant cells. MAb anti-tissue-type plasminogen activator consistently marked endothelial cells in GBM tumor sections but failed to react with tumor cells (data not shown). These data suggest that a coordinated regulation of u-PA and u-PAR synthesis may occur in GBM cells, which would presumably function to enhance pericellular proteolysis.

u-PA mRNA Localizes to the Leading GBM Tumor Edge in the Xenograft Model

To determine whether u-PA played a role in GBM tumor cell invasion of normal brain, the expression of u-PA and u-PAR mRNAs was investigated in an intracerebral SCID mouse model of human GBM tumor. This was performed because most astrocytoma biopsies in our institution are needle biopsies and do not sample the leading tumor edge. The leading tumor edge was defined as an increased density of cells with nuclear anaplasia, including groups of these cells, adjacent to low cellularity brain composed of single cells without obvious nuclear anaplasia. In addition, this leading tumor edge was recog-

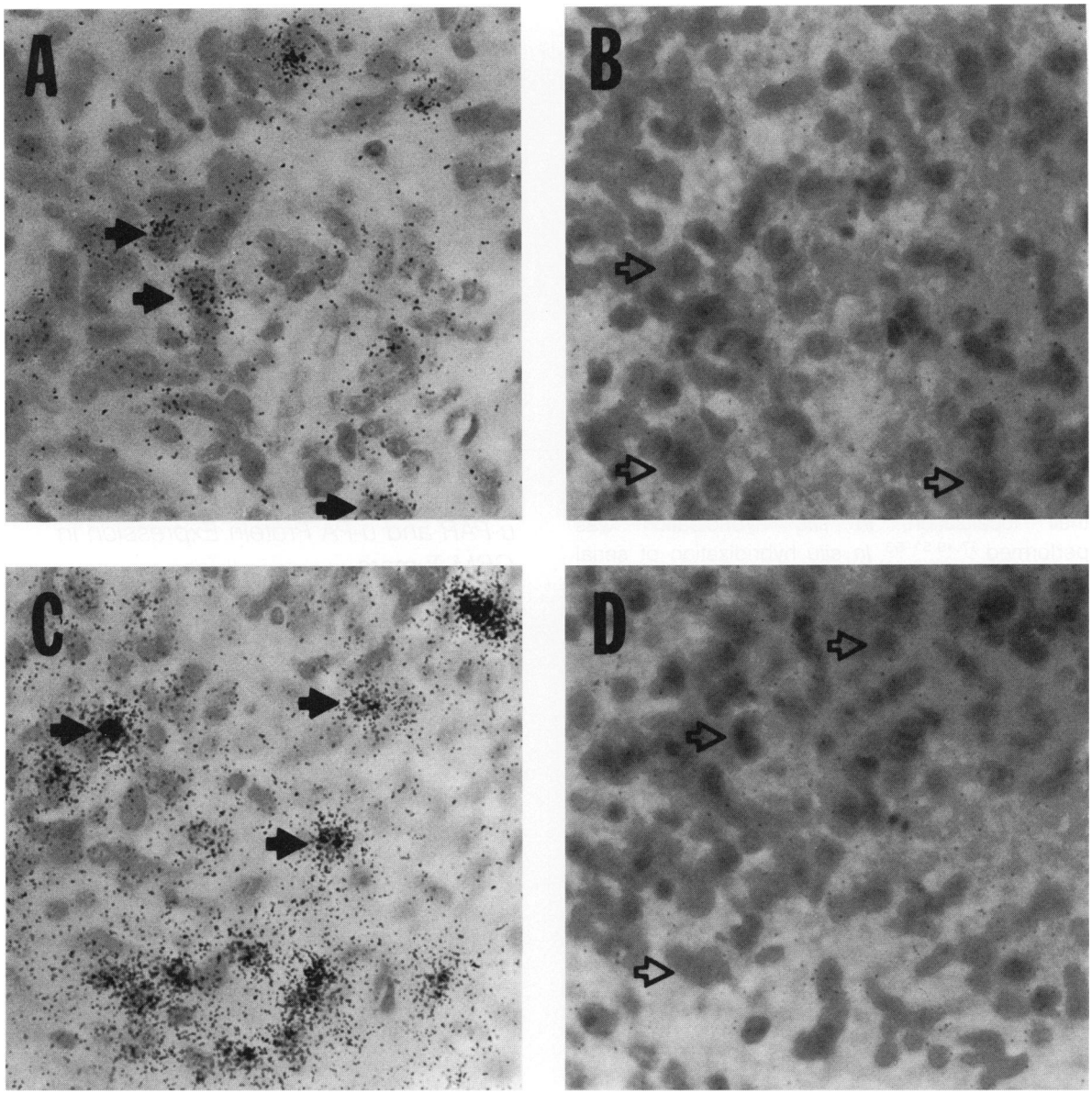

Figure 2. *Expression of both u-PAR and u-PA mRNA marks the malignant astrocyte phenotype. Paraffin sections of brain biopsies were deparaffinized, proteinase K digested, and hybridized with antisense u-PA and u-PAR riboprobes and sense u-PA and u-PAR riboprobes, as described in Materials and Methods.*[49,51–53] *u-PA mRNA expression* (A) *and u-PAR mRNA expression* (C) *localized to malignant astrocytes (closed arrows) in GBM tumors. Sense u-PA and u-PAR riboprobes failed to hybridize to the malignant astrocytes (open arrows) in a GBM tumor* (B *and* D, *respectively). Magnification, ×50.*

nized by a human specific MAb anti-integrin αv subunit (data not shown), as previously published.[49] We cannot rule out the possibility that single invading tumor cells may not be detected by histopathological analysis or MAb anti-integrin αv subunit immunohistochemistry. In this U-251MG intracerebral GBM xenograft model, u-PA mRNA was localized to the leading tumor edge (Figure 4, Panel A), while on serial sections u-PAR mRNA was expressed throughout the tumor (data not shown). Cyclophilin mRNA, a constitutively expressed gene,[50] was expressed homogeneously throughout the tumor (Figure 4B), validating our technique and reflecting on the specificity of the induction of u-PA. Serial sections hybridized with a u-PA sense probe were negative (Figure 4C). In this intracerebral xenograft model, the localization of the u-PA and u-PAR proteins, as analyzed by immunohistochemistry, paralled that of the mRNA expression (data not shown). Co-expression of u-PA and u-PAR in invading cells suggests that focal up-regulation of

Table 1. *Astrocyte Gene Expression of u-PA and u-PAR*

Case	Diagnosis	Sex	Age	u-PA mRNA	u-PAR mRNA
1	Chronic inflammation	M	72	–	–
2	Dysgenesis	M	46	–	–
3	Alzheimer's disease	M	70	–	–
4	Hippocampal dysplasia	F	30	–	–
5	Infarction	F	83	–	–
6	No pathological diagnosis	F	48	–	–
7	Infarction	M	30	–	–
8	Low grade astrocytoma	M	56	–	–
9	Low grade astrocytoma	F	37	–	wk+16%
10	Low grade astrocytoma	M	27	–	–
11	Low grade astrocytoma	M	37	–	–
12	Anaplastic astrocytoma	M	42	–	++ 3%
13	Anaplastic astrocytoma	F	29	–	+ 100%
14	Anaplastic astrocytoma	M	32	–	++ 13%
15	Anaplastic astrocytoma	F	40	–	+ 9%
16	GBM	M	38	++ 25%	++ 22%
17	GBM	F	73	++ 16%	++ 5%
18	GBM	M	61	++ 18%	++ 20%
19	GBM	F	29	++ 9%	++ 60%
20	GBM	M	42	++ 5%	++ 8%
21	GBM	M	56	+ 4%	++ 50%
22	GBM	F	83	++ 17%	++ 2%
23	GBM	F	69	+ 1%	+ 4%

–, <1% positive cells; +, hybridization of antisense probe as described in Materials and Methods. The hybridized sections were graded as positive if ≥4 silver grains were observed over a nucleus or perinuclear area in at least two exposures on two different hybridizations. The grading system was as follows: 4 to 6 grains, weakly positive (wk+); 7 to 12 grains, positive (+); >12 grains strongly positive (++) under ×40 Magnification. The mean percentage of positive cells for u-PAR mRNA in anaplastic astrocytoma is 31 ± 23 (SE); for u-PA and u-PAR mRNAs in GBM tumors, 12 ± 3 (SE) and 21 ± 7 (SE), respectively. Tissues were collected and hybridized with riboprobes as described in Materials and Methods.

the u-PA gene may have important physiological significance; for example, to promote tumor cell invasion.

u-PA and u-PAR Co-Localize on GBM Cells to Sites of Cell-Matrix Contact

To determine whether u-PA and u-PAR co-localize at sites of cell-matrix contact on GBM cells, double-label immunofluorescence with anti-u-PA and anti-u-PAR antibodies was performed on U-251MG cells adherent to vitronectin in serum-free media.[55] u-PA and u-PAR proteins co-localized at sites of cell-matrix contact, known as focal contacts (Figure 5A, B, respectively). u-PA also co-localized with vinculin, a cytoskeletal protein known to localize to focal contacts (data not shown). We have previously reported that U-251MG cell attachment to vitronectin is mediated in part through integrin $\alpha v\beta 3$[40] and that integrin $\alpha v\beta 3$ mediates GBM cell vitronectin-directed migration.[49] In this manuscript we additionally show that the $\beta 3$ integrin subunit (Figure 5D) co-localized with u-PA (Figure 5C) to focal contacts on U-251MG cells, as did u-PA and u-PAR with the αv subunit (data not shown). The $\beta 3$ integrin subunit is thought to pair solely with the αv integrin subunit on nucleated cells.[40,41] These data provide evidence for GBM u-PA binding to u-PAR and localizing to integrin $\alpha v\beta 3$ cell-matrix contacts (focal adhesions).

Discussion

Tumor cell invasion is thought to require at least three steps, ie, tumor cell adhesion, tumor cell protease secretion with subsequent matrix digestion, and tumor cell migration.[4] The serine protease u-PA has been shown to be important in several invasive tumor cell types.[1–3] In fact, studies of other tumors have shown that receptor-bound u-PA is a major determinant of the invasive phenotype *in vivo*,[25] and u-PAR binding enhances u-PA-dependent cellular invasion of the extracellular matrix *in vitro*.[24–27] u-PA activity has previously been reported in extracts of GBM tumors *in vitro*[12,31] and localized to astrocytoma cells *in vivo*[28]; however, expression of astrocyte and astrocytoma cell u-PAR was unknown. In this report, we demonstrate that u-PAR and u-PA mRNAs and proteins are co-expressed in GBM tumor cells, and their co-expression marks the malignant astrocyte phenotype. We also show that u-PA localizes predominantly to the leading tumor edge in the intracerebral SCID mouse GBM xenograft, extending a similar observation of Yamamoto et al[28] in human biopsy tissue. Taken together, these data may indicate

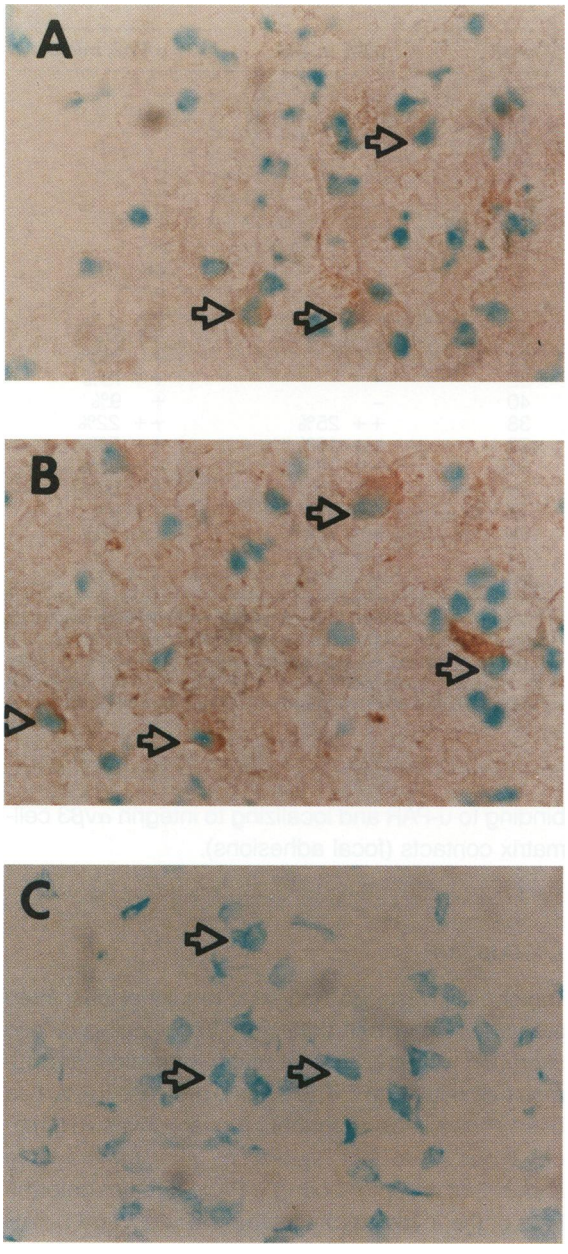

Figure 3. *GBM tumor expression of u-PAR and u-PA proteins. Cryostat sections of GBM human tumor biopsy were reacted with anti-u-PA and anti-u-PA antibodies (10 μg/ml), followed by the immunoperoxidase technique, as described in Materials and Methods.*[13] *u-PA* (A) *and u-PAR* (B) *proteins were detected in malignant astrocytes (arrows) in GBM tumors. Normal rabbit serum IgG controls were negative* (C, *arrows*). *Magnification, ×50.*

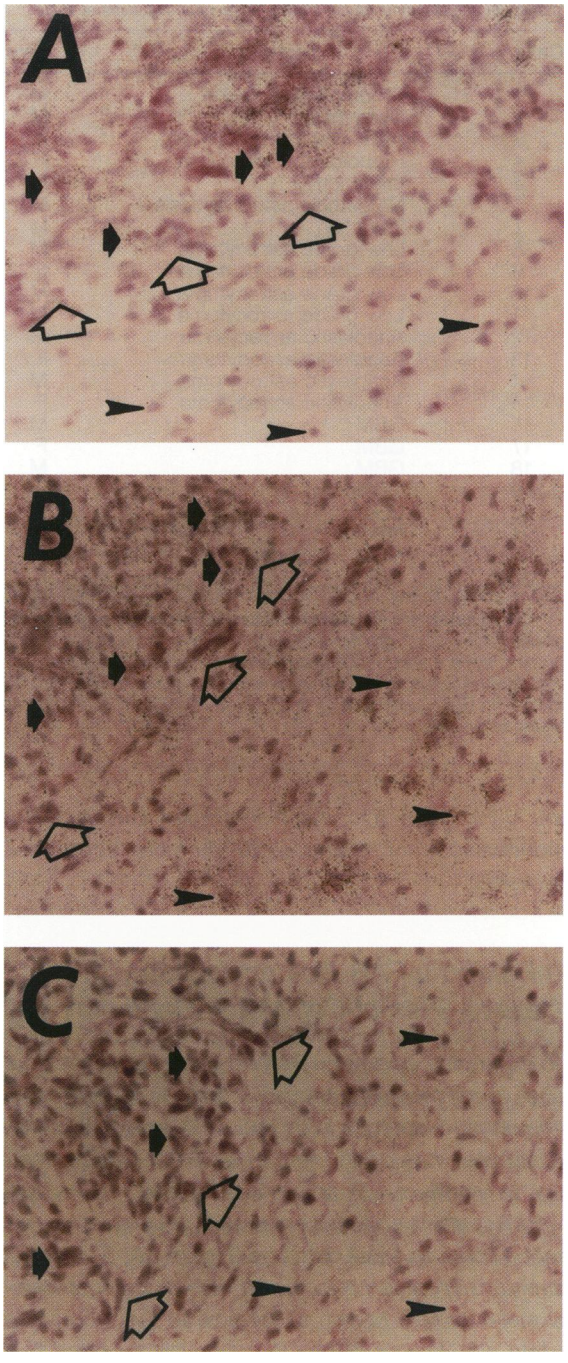

Figure 4. *u-PA mRNA localizes to the leading tumor edge in a SCID mouse xenograft of U-251MG GBM tumor. Cryostat sections of SCID mouse brain xenograft were hybridized with antisense u-PA and cyclophilin riboprobes, as well as sense u-PA riboprobe, as described in Materials and Methods.*[49,51–53] *The leading tumor edge is denoted by open arrows, malignant astrocytes are denoted by closed arrows, and nontumorous glial cells are denoted by arrowheads. Perinuclear and nuclear silver grains were demonstrated over malignant astrocyte nuclei (closed arrows) adjacent to the leading tumor edge when hybridized with antisense u-PA* (A), *whereas nontumorous glial cells failed to hybridize (arrowheads). Antisense cyclophilin riboprobe hybridized to all cells, consistent with expression of a housekeeping gene* (B). *Sense u-PA riboprobe failed to hybridize* (C). *Magnification, ×50.*

a focal regulation of u-PA in invading GBM cells. Lastly, we demonstrate for the first time, to our knowledge, in GBM cells *in vitro*, that u-PA and u-PAR co-localize to integrin $\alpha v\beta 3$ focal contacts, thus providing a mechanism for focal protease activity at cell-matrix contact sites.

Cellular co-expression of u-PA and u-PAR may be a mechanism to facilitate invasion of normal brain, as

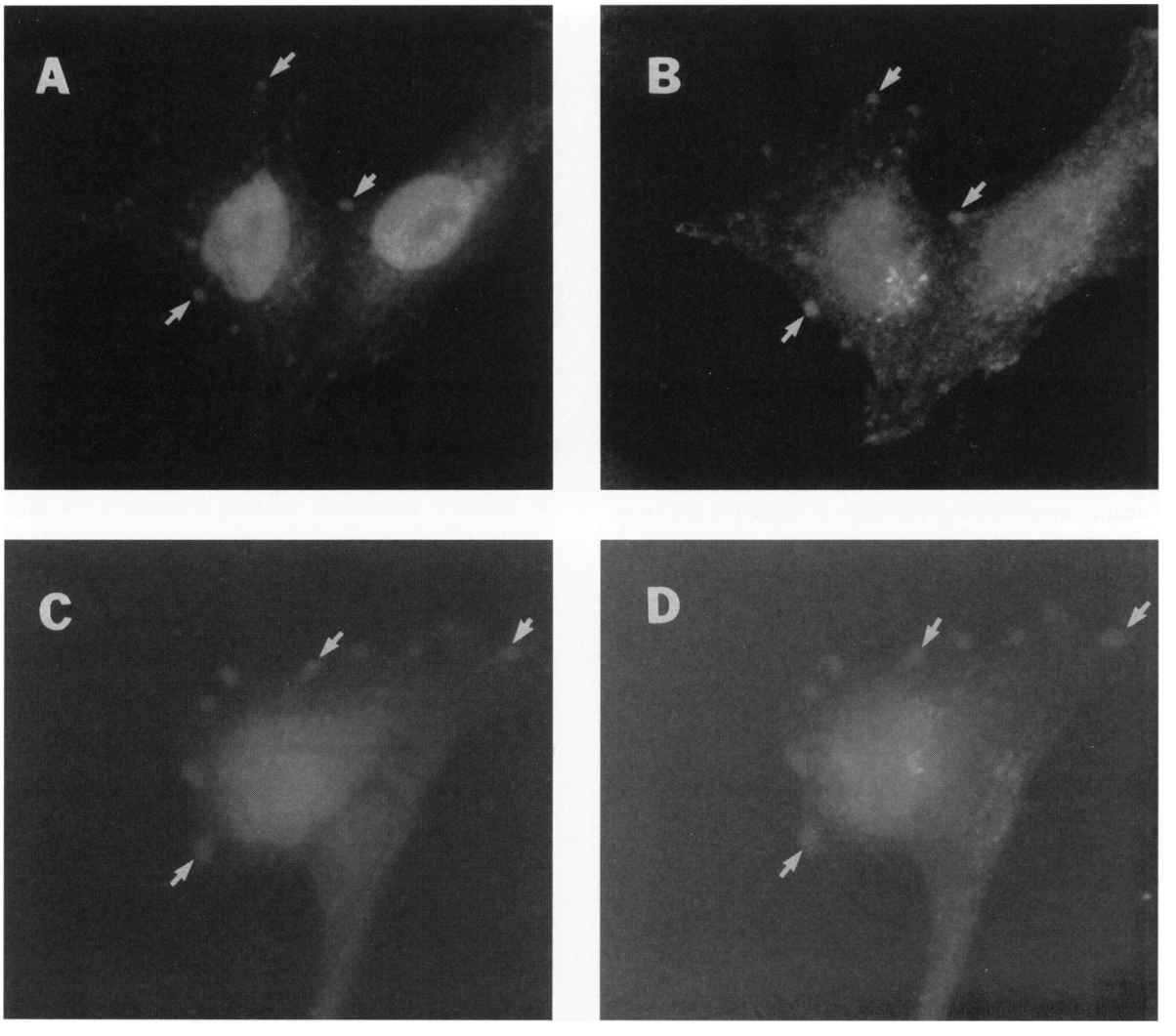

Figure 5. *Co-localization of u-PA and u-PAR proteins with integrin αvβ3 in GBM cells. Double-label (rhodamine and fluorescein) co-localization studies were performed on adherent U-251MG GBM cells in serum-free media, as described in Materials and Methods.*[55] *u-PA* (A) *and u-PAR* (B) *proteins co-localize to the same cell membrane sites (focal contacts). In* C *and* D, *u-PA co-localizes with the β3 integrin subunit, respectively, to focal contacts. u-PA and u-PAR also co-localized with the αv integrin subunit to focal contacts (data not shown). The β3 integrin subunit is thought to pair solely with the αv integrin subunit on nucleated cells.*[40,41,58] *Arrows indicate focal contacts. Magnification, ×125.*

it may achieve focal destruction of normal structures by varying the u-PA activity at focal contact sites on invading cells. This hypothesis is supported by the work of Mohanam et al[26] and others,[9,24,27] in which the number of u-PAR on the tumor cell surface was correlated with tumor cell invasiveness *in vitro*. Our immunohistochemical data on serial human GBM biopsy sections and in the SCID mouse xenograft model suggest that single GBM cells at the leading tumor edge co-express u-PA and u-PAR, thereby participating in local invasion. However, double-labeling techniques will be required to definitively determine this.

We have previously reported that integrin αvβ3 marks the malignant astrocyte phenotype and promotes vitronectin-directed adhesion and migration in GBM cells.[40,49] Our observation of u-PA/u-PAR co-localization with integrin αvβ3 on GBM cells provides a potential link between adhesion and focal extracellular matrix digestion in GBM cells *in vivo*. This focal extracellular matrix destruction may be achieved through integrin-mediated localization of u-PAR to sites of cell-matrix contact or apposition.

In further support of an *in situ* u-PA-dependent proteolytic mechanism in these tumors, the u-PA substrate plasminogen, as well as plasminogen receptors, have been shown to be expressed in adult brain[59] and on GBM cells,[22] respectively. Thus, u-PA bound to u-PAR can result in enhanced plasmin generation on the surface of GBM cells *in vivo*, as has

been reported for other cell types *in vitro*.[3,20,23] In GBM tumors *in vivo*, single-chain u-PA may exist as a latent pool of receptor-bound molecules, itself requiring activation, as has been reported *in vitro*.[60] Single-chain u-PA may be activated by nerve growth factor-γ[61] or plasmin,[2,3] which could then potentially activate single-chain u-PA on the GBM cell surface. Plasminogen could also be available from serum after focal breakdown of the blood-brain barrier,[43] and thus activation of plasminogen to plasmin could occur by tumor cell u-PA or plasma-derived tissue-type plasminogen activator.[1–3] u-PA proteolytic substrates in GBM tumors may be multiple; for example, a recent report indicated u-PA-dependent plasmin proteolysis degrades brain myelin basic protein,[17] a major component of the brain extracellular matrix or neuropil. Plasmin-dependent proteolysis in GBM tumors may also result in degradation of pial/glial and endothelial cell basement membrane laminin, fibronectin, and type IV collagen.[14–16,18] Thus, GBM cells have the capability to promote local tumor invasion into brain parenchyma through u-PA-dependent protease mechanisms, as well as tumor cell spread along the pial/glial and endothelial cell basement membranes. However, u-PA protease activity *in vivo* is likely regulated on multiple levels, for example, through u-PAR expression and/or serine protease inhibitors.[1–3,9–12,15,16,21,23,26,27,30–32]

In summary, we demonstrate the co-expression of u-PAR and u-PA mRNAs and proteins in malignant astrocytes *in vivo*. u-PA and u-PAR co-localization at the leading tumor edge in the intracerebral SCID mouse xenograft and their co-localization with integrin $\alpha v\beta 3$ in GBM cells *in vitro* suggest a biological mechanism for u-PAR-dependent GBM cell migration/invasion *in vivo*. The intracerebral SCID mouse model of human GBM tumor also provides an opportunity in which to further study u-PA-dependent GBM cell invasion, as well as therapies directed at blocking u-PA-dependent GBM cell invasion.

Acknowledgments

Max Williams and Charles Roberts are thanked for their fine technical assistance.

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