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Short communication

Expression of the urokinase plasminogen activator receptor (uPAR) and its ligand (uPA) in brain tissues of human immunodeficiency virus patients with opportunistic cerebral diseases

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The urokinase plasminogen activator receptor (uPAR) and its ligand (uPA) play an important role in cell migration and extracellular proteolysis. We previously described uPAR/uPA overexpression in the cerebrospinal fluid (CSF) and brain tissues of patients with human immunodeficiency virus (HIV)-related cerebral diseases. In this study, we examined uPAR/uPA expression by immunohistochemistry (IHC) in brains of HIV patients with opportunistic cerebral lesions and in HIV-positive/negative controls. uPAR was found in macrophages/microglia with the highest levels in cytomegalovirus (CMV) encephalitis, toxoplasmosis, and lymphomas; in cryptococcosis and progressive multifocal leukoencephalopathy (PML) cases, only a few positive cells were found and no positivity was observed in controls. uPA expression was demonstrated only in a few macrophages/microglia and lymphocytes in all the cases and HIV-positive controls without different pattern of distribution; no uPA immunostaining was found in cryptococcosis and HIV-negative controls. The higher expression of uPAR/uPA in most of the opportunistic cerebral lesions supports their role in these diseases, suggesting their contribution to tissue injury. *Journal of NeuroVirology* (2009) **15,** 99–107.

Keywords: plasminogen activation system; central nervous system; opportunistic infection; HIV; immunohistochemistry

Introduction

The urokinase plasminogen activator receptor (uPAR) and its ligand (uPA) are two key components of the plasminogen activation system (uPAR/uPA system), also including the tissue-type plasminogen activator, the inhibitors plasminogen activator-1, and protease nexin-1. The activation of the uPAR/uPA system leads to cell proliferation, migration, and adhesion, and to extracellular proteolysis by conversion of plasminogen into plasmin, which is involved in the degradation of extracellular matrix protein and basement membranes (Collen, 2001).

In the central nervous system (CNS), plasminogen activation is known to be associated with the breakdown of structural components of the blood-brain barrier (BBB) and thus play a role in the pathogenesis of CNS diseases (Cinque *et al*, 2004; Notter, 1999). In several CNS neoplasms, infections, and neurodegenerative disorders, such as cerebral malaria, multiple sclerosis, and Alzheimer's disease, uPAR has indeed been found to be highly expressed

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in brain tissues (Fauser *et al*, 2000; Deininger *et al*, 2002; Gveric *et al*, 2001; Mohanam *et al*, 1999; Walker *et al*, 2002); moreover, increased levels of suPAR—the soluble form of uPAR that is released from the cell surface—have been detected in cerebrospinal fluid (CSF) of patients with CNS diseases (Akenami *et al*, 1997; Akenami *et al*, 1996; Garcia-Monco *et al*, 2002; Ostergaard *et al*, 2004; Winkler *et al*, 2002).

Based on the hypothesis that the uPAR/uPA system could also be involved in the pathogenesis of human immunodeficiency virus (HIV)-related CNS manifestations, we recently investigated uPAR and uPA expression in the CSF of HIV-infected patients (Cinque *et al*, 2004; Sidenius *et al*, 2004). We found increased suPAR and, to a lesser extent, uPA levels in the CSF of HIV-positive patients, as compared with HIV-negative controls. The highest values were observed in patients with acquired immunodeficiency syndrome (AIDS) dementia complex (ADC), cytomegalovirus ventriculoencephalitis (CMV-VE), and cryptococcosis (Cinque *et al*, 2004; Sidenius *et al*, 2004). We also examined brain tissues with HIV-related lesions (Cinque *et al*, 2004; Sidenius *et al*, 2004), and, preliminarly, tissue samples from a few cases of CMV-VE and cryptococcosis, all showing uPAR staining in macrophages and microglial cells (Cinque *et al*, 2004).

In this study, we systematically examined a large number of cases with different CNS opportunistic infections (CNS-OIs) by immunohistochemistry for uPAR and, in a subset of cases, uPA. We assessed uPAR and uPA tissue distribution, evaluated their degree of expression and defined their cellular origin.

Results

Histological lesions and inflammatory infiltrate

The degree of uPAR and uPA staining was expressed as the number of uPAR/uPA-positive cells out of the number of inflammatory cells, evaluated in each case by histology and immunohistochemistry (IHC).

All the CMV-VE cases were characterized by extensive necrosis, haemorrhage, and a diffuse inflammatory infiltrate consisting predominantly of a high number of macrophages, characterized by increased cell size, abundant cytoplasm, pleomorphic nuclei, and sometimes nucleoli; lymphocytes were few and focally distributed. In addition, a high number of activated microglia (characterized by hypertrophic cell bodies and shorter, thicker processes) and cytomegalic cells, with typical intranuclear and intracytoplasmic inclusion bodies, were present.

In toxoplamosis and PCNSL, histology revealed large foci of necrosis, surrounded by tachyzoites or neoplastic cells, respectively. The inflammatory reaction was abundant and contained a high number of macrophages and lymphocytes. Only a few granulocytes within the central core of the lesions were observed in toxoplasmic encephalitis. Moreover, activated microglial cells bordering the lesions were a constant finding in all the cases.

All the PML cases presented with diffuse demyelinating lesions and inflammatory cells, represented by a high number of foamy macrophages engulfing myelin debris and a few lymphocytes. In addition, viral-infected oligodendrocytes (with basophilic chromatin-deficient nuclei) and ''bizzare'' astrocytes (with enlarged and pleomorphic nuclei) were always observed in association with a few activated microglial cells.

In cryptococcosis, compact aggregates of fungi displaced the leptomeninges and brain parenchima, and formed ''pseudocystic'' lesions. Inflammation consisted on moderate perivascular cuffing of mononuclear cells and a focal collection of a small number of macrophages and lymphocytes. No evident microglial reaction was observed.

uPAR expression

uPAR IHC was positive in 32 of 36 OI cases (Table 1, Figure 1). uPAR expressing cells were detected in all the cases of CMV-VE (13/13, 100%), toxoplasmosis (5/5, 100%), and PCNSL (6/6, 100%), in 4 of 7 PML cases (4/7, 57%), and in 4 of 5 cases of cryptococcosis (4/5, 80%). No uPAR staining was observed in any the HIV-positive and HIV-negative controls.

uPAR expression was always observed in the cell membrane of macrophages and activated microglial cells located within and around the opportunistic lesion; moreover, in the CMV-VE cases, uPAR was present in the cytoplasm of some cytomegalic cells.

The macrophagic/microglial origin of uPAR-producing cells was confirmed by double immunochemical staining with anti-CD68 antibody (Figure 2). No CD3-positive lymphocytes were found to express uPAR antigen.

As the only uPAR-positive cells were macrophages and microglial cells, CD68- but not CD3-positive cells were taken into account for the scoring of uPAR staining. A high grade of uPAR expression (more than 25% of the CD68-positive cells) were found in all the cases except for one PML case and all the uPAR-positive cases with cryptococcosis. In the PML case with low uPAR expression, a high number of macrophages and activated microglial cells were observed in the context of demyelination, as demonstrated by CD68 staining, but only a few macrophages and microglial cells were uPAR positive. In the four cases with cryptococcosis, inflammatory cells were focally distributed and uPAR antibody stained only a few macrophages, some of them phagocyting fungal elements.

Table 1 uPAR and uPA immunohistochemical expression and grading in HIV-positive cases with opportunistic infections and in HIV-positive/HIV-negative controls without cerebral diseases

	uPAR IHC expression			uPA IHC expression		
Cases and controls	High	Low	None	High	Low	None
CMV-VE	13/13	0	0	0	7/7	0
PML	3/7	1/7	3/7	0	4/5	1/5
Toxoplasmosis	5/5	0	0	0	4/4	0
Cryptococcosis	0	4/5	1/5	0	0	1/1
PCNSL	6/6	0	0	0	6/6	0
HIV-positive controls	0	0	15/15	0	6/15	9/15
HIV-negative controls	0	0	3/3	0	0	3/3

Note. IHC: immunohistochemistry; CMV-VE: CMV necrotizing ventriculoencephalitis; PML: progressive multifocal leukoencephalopathy; PCNSL: primary central nervous system lymphoma.

uPA expression

uPA IHC was performed in a subset of cases: 7 CMV-VE, 5 PML, 4 toxoplasmosis, 6 PCNSL, 1 cryptococcosis and in all the HIV-positive and HIV-negative controls. uPA-positive cells were found in all the cases of CMV-VE (7/7, 100%), necrotizing toxoplasmosis (4/4, 100%), and PCNSL (6/6, 100%), in 4 of the 5 PML cases (4/5, 80%), and in 6 of the 15 HIV-positive controls (6/15, 40%). uPA IHC was negative in one case of PML (1/5, 20%), in the cryptococcosis (1/1, 100%), in 9 of the 15 HIV-positive controls (9/15, 60%), and in the 3 HIV-negative subjects (3/3, 100%) (Table 1).

In all the types of OIs, uPA was expressed in the cell membrane of macrophages and microglial cells and in the cytoplasm of T lymphocytes; in PCNSL, uPA staining was found also in the cytoplasm of neoplastic cells. In the HIV-positive controls without CNS disease, uPA antigen was expressed only by rare perivascular lymphocytes. Double IHC confirmed the origin of uPA-positive cells (Figure 2).

uPA-positive cells were distributed within and around the opportunistic lesions and the semiquantitative score was low in all the cases. In detail, if we considered macrophages/microglial cells and lymphocytes separately, less than 25% of the CD68 and CD3 inflammatory cells were uPA positive.

All the cases expressing uPA antigens were also uPAR positive at IHC, with the exception of one PML case, in which only uPA- but not uPAR-positive cells were found.

Discussion

In the present study, we extended our previous observations regarding the expression of uPAR and uPA in brain tissues of patients with OIs. We assessed uPAR and uPA tissue distribution, evaluated thei degree of expression, and defined their cellular origin. In addition, we correlated the tissue expression with our previously published results regarding uPAR/uPA CSF levels (Cinque *et al*, 2004; Sidenius *et al*, 2004) in the same groups of patients.

We found both uPAR and uPA staining in most of the OI cases; conversely, in HIV-positive controls without cerebral disease, we detected only rare uPA-positive cells and no uPAR signal. Finally, HIV-negative controls had no uPAR or uPA staining.

uPAR expression was high in all the cases with CMV-VE, necrotizing toxoplasmosis, and PCNSL, and in three PML cases; on the contrary, uPAR was expressed to a lower extent or not at all in four PML cases and cryptococcosis. In the cases characterized by abundant uPAR expression, the positivity was always found in macrophages and microglial cells within and around the opportunistic lesions, usually with cell membrane localization. uPAR immunostaining localized only a few or no macrophages and microglial cells in four PML cases, despite abundant and diffuse inflammation. In four cryptococcal meningoencephalitis, where the inflammatory reaction was focal, the uPAR antibody stained a very few macrophages phagocyting yeast-like cells.

These findings partly reflect our previously published tissue and CSF observations (Cinque *et al*, 2004). In our previous paper, we showed a high expression of uPAR in brain tissue from patients with HIV-related lesions and CMV-VE and elevated suPAR levels in most of the CNS-OIs compared to HIV-positive patients without CNS disease and HIV-negative controls. More in detail, we found higher CSF levels in CMV-VE and cryptococcosis and lower levels in toxoplasmosis and PCNSL. SuPAR levels in PML cases were very low and they did not differ from those of HIV-positive cases without cerebral involvement (Cinque *et al*, 2004).

SuPAR, the soluble form of uPAR, is generated by hydrolysis of glycosylphosphatidylinositol (GPI)-anchored membrane uPAR (Brunner *et al,* 1999); so, CSF suPAR levels are expected to depend on the number of uPAR-expressing cells in the CNS and the release rate of uPAR to form suPAR (Cinque *et al*, 2004).

This hypothesis is supported by the finding of high uPAR/suPAR expression in brain tissues and CSF of patients with CMV-VE and ADC, indicating

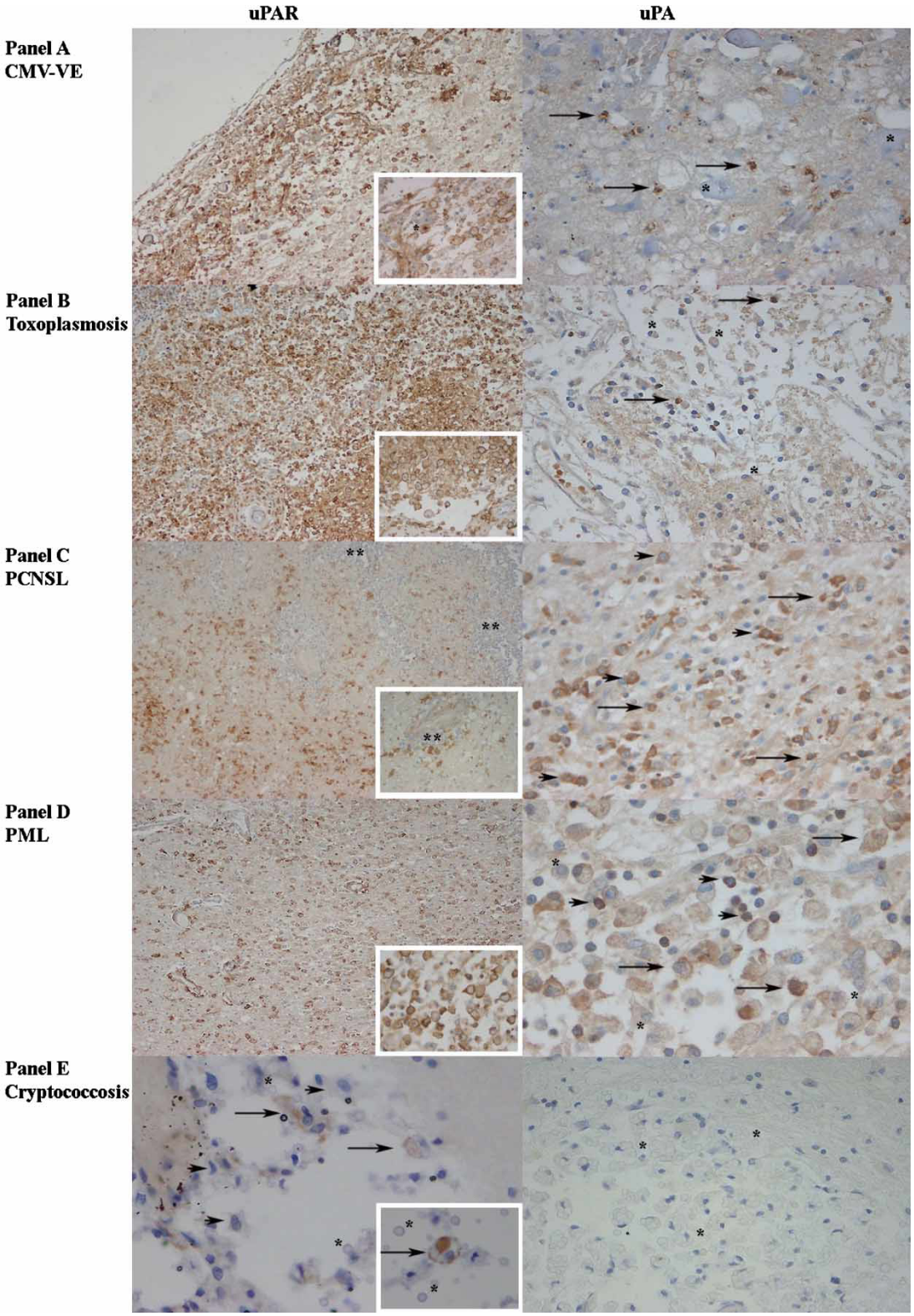

Figure 1 (*Continued*)

that macrophages/microglial cells could be the main source of suPAR in these cases (Cinque *et al*, 2004, and present paper). We previously demonstrated that in patients with HIV-related brain lesions and ADC, the sources of uPAR in brain tissues were both HIV-infected microglial cells and reactive macrophages (Cinque *et al*, 2004). The role of macrophages/microglial cells in the pathogenesis of ADC is well known; thus, an association between HIV and uPAR in the CNS can be suggested. In addition, HIV up-regulates monocytic uPAR expression *in vitro* (Speth *et al*, 1998) and its functional interactions with the uPAR/uPA system have been demonstrated (Alfano *et al*, 2002; Wada *et al*, 2001).

CSF suPAR levels in PCNSL and toxoplasmosis were found to be relatively low (Cinque *et al*, 2004) compared to abundant tissue expression, as we described. It is thus possible hypothesize that there is a low rate of suPAR release from macrophages and activated microglial cells in these cases.

In addition, there is indeed an apparent discrepancy between low uPAR brain expression and elevated CSF suPAR in cryptococcosis. The correlation between CSF suPAR, mononuclear cell counts and HIV-1 load, as we previously reported, suggests that peripheral mononuclear cells trafficking within the CSF could be the source of CSF suPAR in these cases (Cinque *et al*, 2004).

Regarding uPA, we previously examined tissue expression and CSF levels in patients with HIV-related brain lesions, CMV-VE, and in HIV-positive and -negative controls without cerebral involvement (Sidenius *et al* 2004). We found uPA CSF levels more than twofold lower than suPAR levels. However, the distribution of uPA CSF values between patient groups was similar to that of suPAR, with higher uPA concentrations in patients with brain lesions compared to HIV-positive and -negative subjects (Sidenius *et al* 2004). By IHC, uPA antigens were found in mononuclear cells, both isolated and in microglial nodules, and in HIV-related multinucleated giant cells. The number of uPA-positive cells was in all the cases lower than the number of both HIV-p24– and uPAR-positive cells.

In the present study, we extended the analysis of uPA expression in brain tissues by IHC. Differently from uPAR, uPA had a low score of expression in all the positive cases, always limited to a small number of macrophages/microglial cells and lymphocytes. In addition, the pattern of uPA immunostaining did not differ between patient groups. We also observed the presence of a small number of uPA-positive cells in six HIV-positive controls without brain disease, staining negative for both HIV-p24 and uPAR. Interestingly, in the present work we tested also brain tissues from HIV-negative subjects without cerebral lesions and no uPA- or uPAR positive cells were found.

Our findings support the hypothesis that uPA might play a role as down-modulator of HIV, inducing an inhibitory signal for HIV replication following binding to cell surface suPAR, and suPAR acts as an enhancer via binding and removal of uPA (Wada *et al* 2001; Alfano *et al* 2002). According to this model, uPA production could be enhanced in brain of patients with HIV infection, with inhibitory effect on HIV replication itself (Alfano *et al*, 2002). In the presence of productive HIV infection (as in HIV-related brain lesions), the increased production of uPAR and suPAR might prevent uPA binding to the cell surface, thus exerting its protective function towards HIV replication. Because the concentration of suPAR in CSF is largely exceeding the concentrations of uPA (Sidenius *et al* 2004), it is possible that suPAR sequesters most of the extracellular uPA, thus preventing its binding to cell surface uPAR. Such a scavenger effect of suPAR could explain the relative low staining for uPA in HIV-related brain lesions, as we previously described.

This model might also apply to CNS-OIs, especially those characterized by elevated suPAR production, such as CMV-VE. Indeed, like in HIV-related lesions, macrophages appear crucial in the pathogenesis of CMV-VE, as shown by histopathological studies and by the finding of very high CSF levels of markers of macrophage activation, such as the monocyte chemotactic protein-1 or β_2-microglobulin (Cinque *et al*, 1998; Sidenius, data not shown). More in general, however, it may not be possible to design a common model for the involvement of the uPA system in CNS-OIs, because these include a spectrum of different infections and neoplasms and, additionally, experimental data or *ex vivo* findings on individual opportunistics are lacking. A number

Figure 1 Immunohistochemical expression of uPAR/uPA in paraffin-embedded tissues from HIV-infected patients with opportunistic cerebral disease. (*Left panels*) uPAR immunohistochemistry. (**A**–**D**) uPAR immunohistochemistry in CMV-VE, toxoplasmosis, PCNSL, and PML cases: A high number of uPAR-positive cells (brown staining) located within and around the opportunistic lesions was found. *Inserts:* High magnification of uPAR positivity in the cell membrane of macrophages and microglial cells. In the CMV-VE cases (**A**), uPAR was present in the cytoplasm of some cytomegalic cells (*). No positivity was found in the neoplastic cells of PCNSL cases (**D**, **). (**E**) uPAR immunohistochemistry in cryptococcosis: Inflammatory cells were distributed in meningeal spaces (*short arrows*) but only few macrophages (*arrows*), some of them phagocyting fungal elements (*), expressed uPAR antigens (brown staining). (*Right panels*) uPA immunohistochemistry. (**A**–**D**) uPA immunostaining in CMV-VE, toxoplasmosis, PCNSL, and PML cases: uPA expression in the cytoplasm of rare lymphocytes (*arrows*) and in the cell membrane of macrophages (**C**, *). In PCNSL, uPA antigens were demonstrated also in the cytoplasm of neoplastic cells (*short arrows*). The majority of inflammatory cells did not express uPA antigens (*). (**E**) No uPA-positive cells were found in cryptococcosis. * indicates macrophages phagocyting fungi. Immunohistochemistry: DAB, hematoxylin counterstaining. Original magnification: ×10 (uPAR, **A**–**D**), ×20 (uPAR *inserts*, **A**–**D**), ×40 (uPAR, **E** and *insert*; uPA, **A**–**E**). This Figure is reproduced in colour in *Journal of Neurovirology* online.

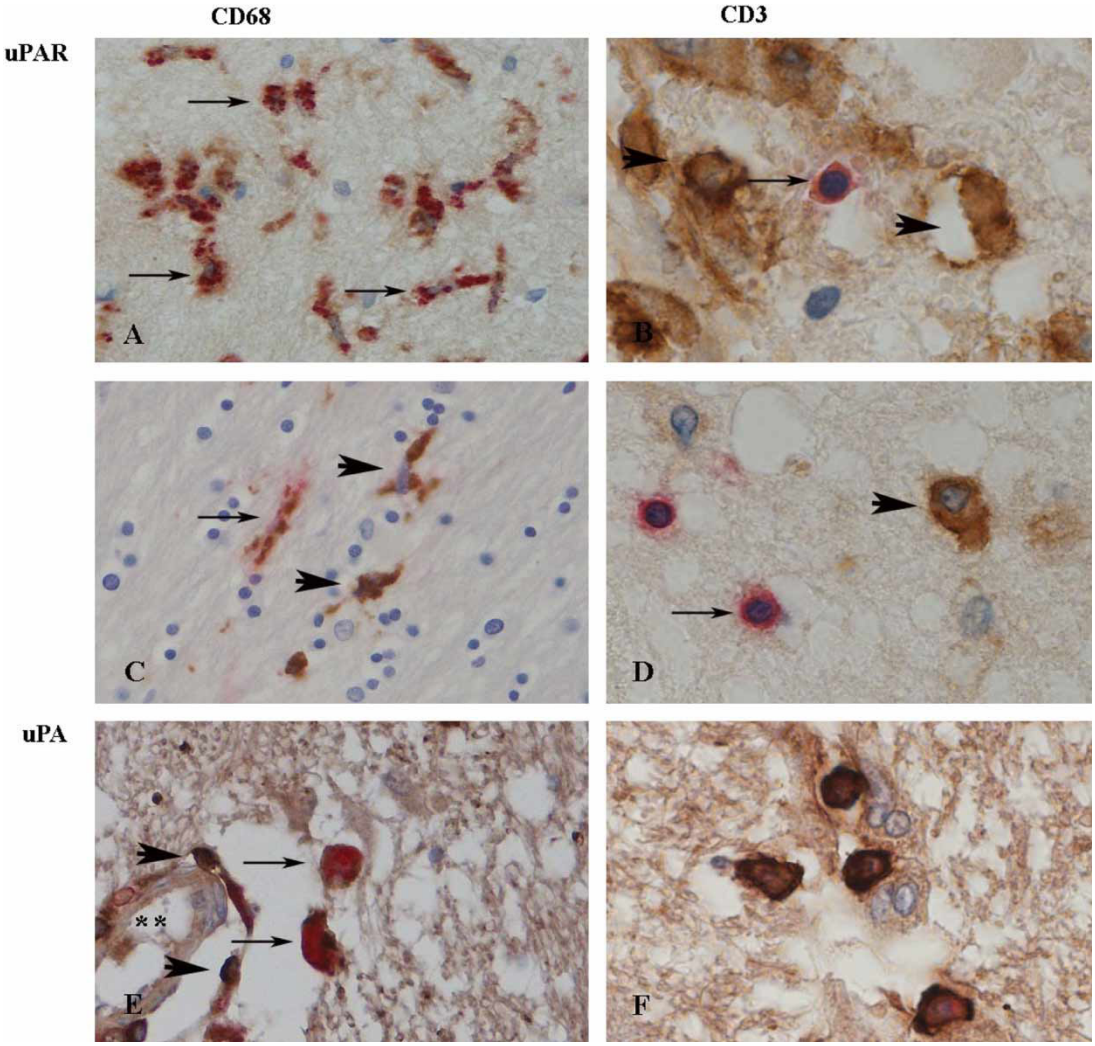

Figure 2 Cellular types producing uPAR and uPA antigens. (**A, C, E**) Double immunostaining with anti-uPAR/uPA (red staining) and anti-CD68 (brown staining) antibodies: The macrophagic/microglial origin of uPAR/uPA-producing cells was demonstrated by the colocalization of the two antigens (*arrows*) in cases of CMV (**A** and **E**) and toxoplasmic (**C**) necrotizing encephalitis. In **C** and **E**, some CD68-positive microglial cells (brown staining, *short arrow*), with ramified cytoplasm and small nuclei, did not express uPAR. ** indicated a blood vessel. (**B, D, F**) Double immunostaining with anti-uPAR/uPA (brown staining) and anti CD3 (red staining) antibodies: No uPAR expression by CD3-positive T lymphocytes (*arrows*) in cases of CMV (**B**) and toxoplasmic (**D**) necrotizing encephalitis. uPAR-positive macrophages/microglial cells were stained in brown (*short arrows*). In **F**, the uPA expression by CD3-positive T lymphocytes was demonstrated by the colocalization of the two antigens (brown- and red-stained cells) in a case of toxoplasmic necrotizing encehalitis. Double immunohistochemistry: DAB and Vulcan Fast Red, hematoxylin counterstaining. Original magnification $\times 40$ (**A, C, F**), $\times 100$ (**B, D, F**). This Figure is reproduced in colour in *Journal of Neurovirology* online.

of studies have shown increased expression and release of uPAR in monocytic cells in response to bacteria and bacterial components, such as endotoxin, lipopolysaccharide (LPS), and bacterial-specific proteins (Coleman *et al*, 2001; Dekkers *et al*, 2000; Ostrowski *et al*, 2005), suggesting a role of this molecule in the pathogenesis of bacterial infections. On the other hand, microbial products might induce proteolytic cleavage of uPAR, leading to reduced cell capacity to bind uPA (Leduc *et al*, 2007). Increased suPAR production and/or the formation of suPAR fragment with chemotactic activity, such as the D2-D3 fragment, generated by proteolytic cleavage of uPAR (Leduc *et al*, 2007) might contribute to recruitment of leukocytes to the CSF, as demonstrated in experimental pneumococcal meningitis in uPAR-deficient mice (Paul *et al*, 2005). Thus, one may speculate that opportunistic agents in HIV infections may similarily interfere with the physiological functioning of uPAR.

Of note, the observed lack of suPAR expression in neoplastic cells in cases with brain lymphoma was consistent with findings in systemic B-cell lymphomas and leukemias. Although tumor cells have been shown to express high uPAR levels in a number of human cancers, uPAR was expressed significantly only by myeloid and monocytic, but not lympoid malignancies (Plesner *et al*, 1994).

Although our tissue findings show different patterns of expression for suPAR and uPA in the different CNS-OIs, these do not allow us to draw any conclusion on the mechanisms by which the uPAR/uPA system interacts with the different pathogens and is involved in tissue damage. For the role of the uPAR/uPA system in the pathogenesis of HIV encephalitis, two main mechanisms have been hypothesised. First, uPAR binding to uPA might accelerate plasmin generation, leading to enhanced remodelling of extracellular matrix and, possibly, to alterations of the brain barriers. In addition, uPAR may induce cell adhesion/migration through its interaction with extracellular matrix protein vitronectin (Madsen *et al*, 2007), whereas the D2-D3 fragment of suPAR can bind to the G protein–coupled receptor FPRL1, resulting in the generation of a chemotactic signal (Resnati *et al*, 2002) and promoting the recruitment of inflammatory cells into the brain. The presence in brain tissues of products of proteolysis would help support the first hypothesis. On the other hand, the recent findings in experimental pneumococcal meningitis seem to confirm the chemotactic function of suPAR although it remains to verify if suPAR fragments may have similar or enhanced chemotactic activity *in vivo* (Paul *et al*, 2005).

In conclusion, our results support the role of the uPA system in most of CNS opportunistic infections. The good correlation between CNS and previous CSF findings indicates that CSF uPAR/uPA levels reflect the expression of these molecules in the brain and represent additional markers to study cerebral pathological conditions.

Material and methods

Brain tissues from 36 HIV-positive patients with histologically documented CNS-OIs were retrospectively examined: 13 cases of extensive necrotizing CMV-VE, 7 of progressive multifocal leukoencephalopathy (PML), 5 of necrotizing toxoplasmosis, 5 of cryptococcal meningoencephalitis, and 6 of primary CNS lymphoma (PCNSL). Autopsies were performed at L. Sacco Hospital, Milan, Italy, during a period of 10 years (1990 to 1999).

The criteria for the inclusion in the study were no antiretroviral drug during the 6 months preceding death and, to minimize the effects of confounding conditions, no systemic diseases affecting brain functioning, as liver failure, hypoxia, metabolic abnormalities.

The cases were 29 males and 7 females; their median age was 36.6 years (range 23 to 68). Their mean CD4 count was 11 cells/microliter (range 2 to 70 cells/μl); the risk factor was intravenous drug abuse in 31 cases and heterosexual/homosexual transmission in 5 cases. Six cases (five with CMV-VE and one with toxoplasmosis) had CMV infection of adrenal glands; two cases (one with toxoplasmosis and one with PML) had extracerebral lymphoma.

The diagnosis of CNS-OIs was performed by histo- and immunochemical stainings (periodic acid Schiff-PAS, Giemsa, Groccot-Gomori, mono- and polyclonal antibodies directed against Toxoplasma *gondii*, CMV, and leukocyte antigens for immunophenotyping of PCNSL).

HIV-related lesions (HIV encephalitis or HIV leukoencephalopathy, established on the basis of the presence of microglial nodules or myelin pallor, both in association with multinucleated giant cells, and HIV-p24 antigen–positive cells at immunohistochemistry) (Nebuloni *et al* 2000) were found in 12 cases, including 8 CMV-VE, 2 PML, and 1 case each of toxoplasmosis and PCNSL.

However, HIV-related and opportunistic lesions involved different areas of the brain and no HIV-positive cells were found in sections chosen for uPAR/uPA immunostaining.

Fifteen HIV-positive (who died of noncerebral events) and three HIV-negative patients (who died of myocardial infarction) were chosen as controls. They were 15 males and 3 females; their median age was 34 years (range 30 to 54) for HIV-positive patients and 73 years (range 69 to 78) for HIV-negative subjects; none of them had CNS disease at histological or immunohistochemical examination.

Regarding HIV-positive controls, the criteria for the inclusion in the study were the same as described for the cases with OIs. In addition, their mean CD4 count was 14 cells/μl (range 10 to 63 cells/μl); the risk factor was intravenous drug abuse in 14 cases and heterosexual/homosexual transmission in 1; 2 of them had CMV infection of adrenal glands.

Haematoxylin-eosin sections from at least seven formalin-fixed, paraffin-embedded cerebral samples were reviewed in each case (frontal, parietal and temporal lobes, basal nuclei, deep white matter, cerebellum, spinal cord, and each macroscopic lesion).

Immunohistochemistry (IHC)

The area with the most representative histological lesion was chosen for IHC. In control groups, IHC was perfomed on a sample from basal nuclei.

Paraffin-embedded sections were cut and mounted on Super-frost slides (Bio-Optica, Milan, Italy). After dewaxing in xylene and rehydrating in ethanol, the sections were pretreated in a microwave oven (two cycles for 5 min each at 780 W, in 0.01 M citrate buffer) and incubated with rabbit anti-human uPAR antibody (raised in our laboratory; 6.3 mg/ml, 1:500 dilution) and rabbit anti-human uPA antibody (raised in our laboratory; 5.7 mg/ml, 1:1000 dilution) for 2 h at room temperature (Sidenius *et al* 2004).

To evaluate the cellular component of the inflammatory infiltrate, the following antibodies were

used: mouse anti-human CD3 (clone PS1; BioGenex, USA; 1:100 dilution, for T lymphocytes) and mouse anti-human CD68 (clone PGM1; DakoCytomation, Denmark; 1:500 dilution), directed against a fixative-resistant epitope on the macrophage-restricted form of the CD68 molecule. The clone CD68 PGM1 is expressed by phagocytic macrophages of microglial and monocytic origin and by ''activated'' microglia.

The reactions were revealed by nonbiotin peroxidase detection system (Supersensitive Non-Biotin Detection System, BioGenex, USA) with 3,3′-diaminobenzidine free base (DAB) as chromogen.

Negative controls were obtained by omission of the primary antibody.

Typing of uPAR/uPA-producing cells

To demonstrate the cellular type producing uPAR and uPA antigens, double immunohistochemical staining was performed by using the antibodies cited above. Specific secondary antibodies conjugated with peroxidase and alkaline phosphatase were chosen (MACH 2 Double Stain 2; BioCare Medical, USA): the first reaction was developed by using DAB as chromogen (brown staining) and the second by using Vulcan Fast Red (red staining).

Cell count

uPAR/uPA expression and the inflammatory cells were evaluated on consecutive sections. The most highly reactive 1-cm^2 area was chosen and each imunostaining was quantify by counting positive cells in five 20 × fields. CD3-positive T lymphocytes and CD68-positive macrophages/microglial cells were considered separately and a semiquantitative three-grade scale was applied: no CD3- or CD68-positive cells, a small number or a high number of CD3- or CD68-positive cells. The quantification of uPAR/uPA expression was based on the number of uPAR/uPA-positive cells out of the number of T lymphocytes (CD3-positive cells) and/or macrophages/microglial cells (CD68-positive cells): no expression corresponding to absence of uPAR- or uPA-positive cells, low expression, corresponding to samples with uPAR- or uPA-positive cells $<25\%$ of CD3- and CD68-positive cells and high expression, corresponding to samples with uPAR- or uPA-positive cells $>25\%$ of CD3- and CD68-positive cells.

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