Interaction of α-2-macroglobulin and HSV-1 during infection of neuronal cells

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We describe the effect of pretreatment with alpha-2-macroglobulin (A2M) on the susceptibility of the human neuroblastoma SKNMC cell line to infection by herpes virus type 1 (HSV-1). ELISA and co-immunoprecipitation experiments confirmed the A2M-HSV-1 interaction *in vitro*. Indirect immunofluorescence shows that A2M exacerbated the cytopathic effect induced after HSV-1 infection. However, A2M-pretreated SKNMC cells notably produced fewer HSV-1 particles than did the untreated cells, suggesting that A2M could induce a restrictive infection. Furthermore, high levels of HSV-1 and A2M induced the production of nitric oxide (NO) in SKNMC. Preliminary results suggest that A2M might induce apoptosis in HSV-1-infected cells. These findings affirm the conclusion that A2M may interact directly with HSV-1 and modulate the course of the infection in SKNMC human neuroblastoma cells. *Journal of NeuroVirology* (2001) **7,** 556–563.

Keywords: α-2-macroglobulin; HSV-1; SKNMC; neuroblastoma; apoptosis

Introduction

Following primary infection of mucosal epithelial cells, herpes simplex virus type 1 (HSV-1) can infect peripheral sensory neurons reaching, under certain conditions, the central nervous system (CNS) (Immergluck *et al*, 1998). Entry of HSV-1 into cells occurs in two sequential steps: i. Binding of virion glycoprotein C (gC) and sometimes gB to cell surface heparan sulfate and then binding of gD to a specific cell receptor (Shukla *et al*, 1999). ii. Fusion of this virion envelope with cell membrane, requiring virus glycoproteins gB, gD, gH and gL (Geraghty *et al* 1998; Shukla *et al*, 1999). Sensory neurons seem to be the main site of HSV latent infection (for review see Jones, 1999). In fact, after reactivation in the CNS, human HSV-1 is responsible for a variety of disease states, including encephalitis and some chronic and progressive neurodegenerative disorders (Itzhaki *et al*, 1997, 1998; Leissring *et al*, 1998; Qiu and Abdel-Meguid, 1999; Cribbs *et al*, 2000). Never-

theless, the molecular mechanism underlying these processes remains obscure.

The human alpha-2-macroglobulin (A2M) is one of the major plasma proteins, representing approximately 8% of the total glycoprotein in the serum (Dunn and Spiro, 1967; Borth, 1992). A2M and related proteins have been described as being elements of the humoral defence barriers of vertebrates, binding different toxins and pathogens such as bacteria or virus (Borth, 1992). In fact, in some species A2M is a pivotal factor that protects against some virus infections (Pritchett and Paulson, 1989). Furthermore, A2M interacts with almost any protease and has been widely believed to function as an important panprotease inhibitor (Harpel, 1973). A2M also serves as a carrier of various nonproteolytic proteins, including cytokines, hormones, and growth factors (Krimbou *et al*, 1998). The exact role of the interaction with these nonproteolytic proteins remains unknown. Moreover, this association is known to be distinct from that between A2M and proteases, which causes the activation of the former, mediated by conformational changes, and the formation of a stable A2M-protease complex. Synthesis of A2M can be carried out by numerous cell types, including hepatocytes, astrocytes, and fibroblasts (Sottrup-Jensen, 1989) and its specific receptor has

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been identified in the aforementioned cell lineages as well as on smooth muscle cells and neurons (Ganter *et al*, 1991; Moestrup, 1994).

In the present report, we have addressed the matter of whether A2M interacts with HSV-1. These studies has been precipitated by the fact that both A2M and HSV-1 could be present in the CNS and have been implicated in neurodegenerative pathologies (Bishop and Hill, 1991; Itzhaki *et al*, 1997, 1998; Blacker *et al*, 1998; Corder *et al*, 1998; Leissring *et al*, 1998; Dow *et al*, 1999; Rudrasingham *et al*, 1999; Singleton *et al*, 1999; Cribbs *et al*, 2000; Krüger *et al*, 2000). Although results are still controversial, A2M seems to play a role in the accumulation and clearance of soluble β-amyloid peptide (Du *et al*, 1998; Fabrizi *et al*, 1999; Qiu *et al*, 1999). Furthermore, A2M (Strauss *et al*, 1992; Blacker *et al*, 1998; Krimbou *et al*, 1998; Dow *et al*, 1999; Singleton *et al*, 1999), as well as HSV-1 (Itabashi *et al*, 1997; Corder *et al*, 1998; Leissring *et al*, 1998), have been associated with apolipoprotein E (apoE), this interaction having been implicated in some chronic neurodegenerative processes, such as Alzheimer's disease. In addition, both apoE and HSV-1 bind to cellular heparan sulfate proteoglycans (Ji *et al*, 1993; Immergluck *et al*, 1998) and might compete for this interaction (Itzhaki *et al*, 1997), which affects the potential interaction of apoE with A2M (Krimbou *et al*, 1998).

We have observed that A2M binds "*in vitro*" HSV-1 particles. Moreover, A2M aggravates the cytopathic effect (CPE) induced by HSV-1 in the human neuroblastoma SKNMC cell line, although the production of new viral progeny decreased after A2M pretreatment. On the other hand, A2M increased the detection of the gas nitric oxide (NO), which is induced after HSV-1 infection. Nevertheless, the treatment solely with A2M failed to induce NO accumulation. Finally, preliminary results suggest that A2M treatments commit SKNMC to apoptosis, independently of HSV-1 infection.

Results

A2M binds to HSV-1 in vitro

A2M is a panprotease inhibitor and carrier of various nonproteolytic proteins, able to interact with an increasing number of substrates (Harpel, 1973). Furthermore, recent information showed evidence for controlled incorporation of HSV-1 protease into the capsid (Sheaffer *et al*, 2000). Taking this into account and also the fact that both A2M and HSV-1 can be present in the CNS and have been involved in neurodegenerative anomalies (Strauss *et al*, 1992; Itabashi *et al*, 1997; Blacker *et al*, 1998; Corder *et al*, 1998; Krimbou *et al*, 1998; Leissring *et al*, 1998; Dow *et al*, 1999; Rudrasingham *et al*, 1999; Singleton *et al*, 1999), we wondered if A2M could be associated with HSV-1 particles. For this purpose, 500 or 0.5 pg of A2M were coated onto 96-well plates and the binding of HSV-1 particles (3×10^6 or 3×10^5 PFU) was studied by ELISA. Figure 1 shows the specific dose-dependent binding of HSV-1 to the protease inhibitor. Similar results were obtained when HSV-1
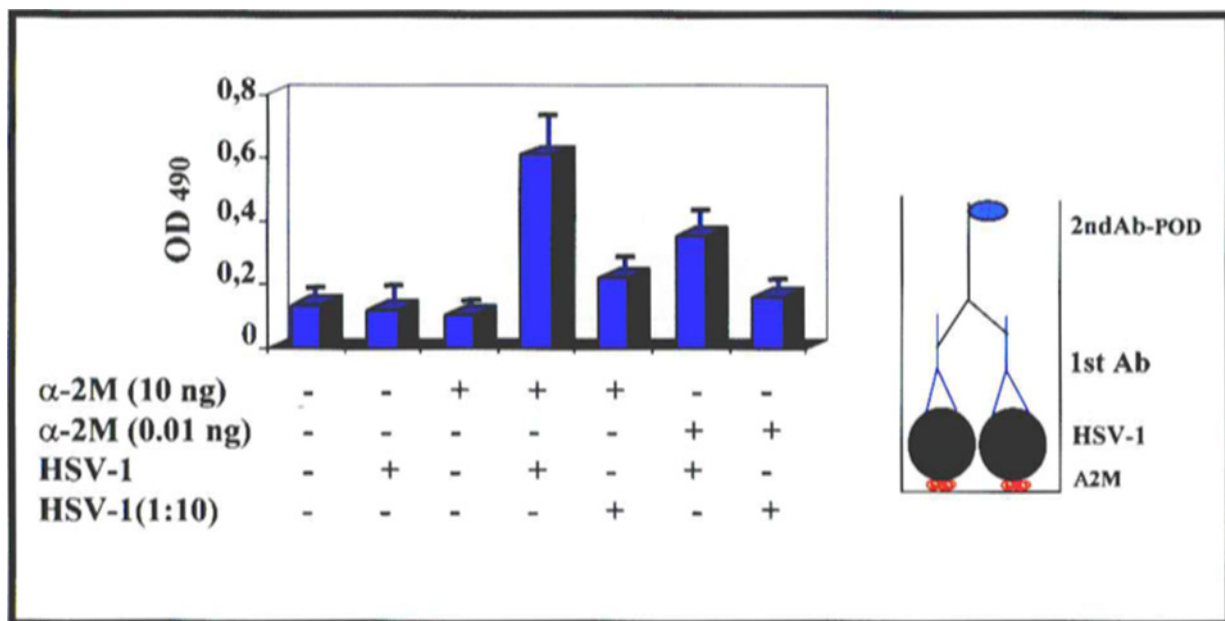

Figure 1 Binding of HSV-1 particles to A2M molecules *in vitro*. Indicated amounts of A2M were coated on 96-well plates and incubated with 3×10^6 (HSV-1) or 3×10^5 (HSV-1 1:10) PFU purified virus particles. The HSV-1 bound was estimated by an ELISA with rabbit polyclonal anti-HSV-1 and goat anti-rabbit immunoglobulins coupled to horseradish peroxidase as described in the Materials and methods. Bars indicate the standard error of the mean. A scheme of the method is shown.
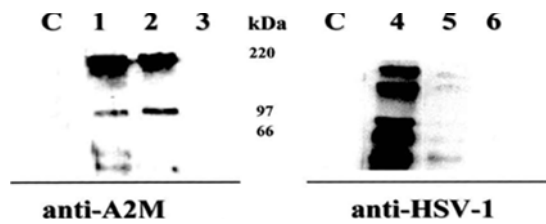
Figure 2 Immunoprecipitation of HSV-1 bound to A2M. HSV-1 (6×10^6 PFU) were incubated with A2M (10 μg) *in vitro* for 1 h at 37°C and immunoprecipitated with a rabbit polyclonal anti-A2M or irrelevant antibodies. Immunoprecipitates were separated by electrophoresis and Western blotting was performed with anti-A2M or anti-HSV polyclonal antibodies. (**C**) A2M + HSV-1 immunoprecipitated with irrelevant antibody. **1** and **4**. Control of purified A2M or HSV-1 alone immunoprecipitated directly with anti-A2M or anti-HSV-1, respectively. **2** and **5**. A2M + HSV-1 immunoprecipitated with anti-A2M. **3**. HSV-1 immunoprecipitated with anti-A2M. **6**. A2M immunoprecipitated with anti-A2M.

Figure 3 Effect of A2M on the infection of SKNMC by HSV-1. Cells were pretreated or not with 150 μg/ml A2M and/or heparin (Hp) at 100 μg/ml. Subsequently, cells were mock-infected or infected with HSV-1 at 1 PFU per cell. Then, 24 h p.i., cells were fixed and immunofluorescence was carried out with a polyclonal anti-HSV-1 antibody.

particles were coated onto the plates and the binding of A2M measured (data not shown). Higher concentration of A2M did not significantly alter the detected signal (data not shown). To validate this finding, the interaction of A2M and HSV-1 was studied by immunoprecipitation and reciprocal Western blot detection (Figure 2). Incubations of A2M and HSV-1 were immunoprecipitated using anti-A2M polyclonal antibodies. Subsequently, precipitation of A2M or HSV-1 was analyzed by Western blot (Figure 2). High A2M levels, similar to that observed by immunodetection of purified A2M, were detected (Figure 2, left). As expected from Figure 1, significant levels of HSV-1 proteins were detected when an anti-HSV-1 polyclonal antibody was used (Figure 2, right). Unspecific immunoprecipitation of HSV-1 or A2M particles was not detected. These results suggest that A2M could directly interact with HSV-1, although the mechanism of that interaction requires further investigation.

Effect of A2M on HSV-1 infection
It was of pivotal interest to determine whether A2M could modulate the course of HSV-1 infection. The human neuroblastoma SKNMC cell line was infected with one PFU per cell HSV-1. Accumulation of viral proteins could be observed 24 h p.i. by immunofluorescence with an anti-HSV-1 polyclonal antibody (Figure 3). However, as previously reported (Immergluck *et al*, 1998), treatment with heparin completely blocked viral protein detection. On the other hand, when cultures were pretreated with A2M, a stronger fluorescence signal was detected. Furthermore, A2M was able to reverse partially the inhibition exerted by heparin, suggesting that A2M increases the synthesis of viral protein in SKNMC. Nevertheless, when the production of new viral particles was analyzed by plaque assay, surprisingly, A2M inhibited the formation of infectious HSV-1
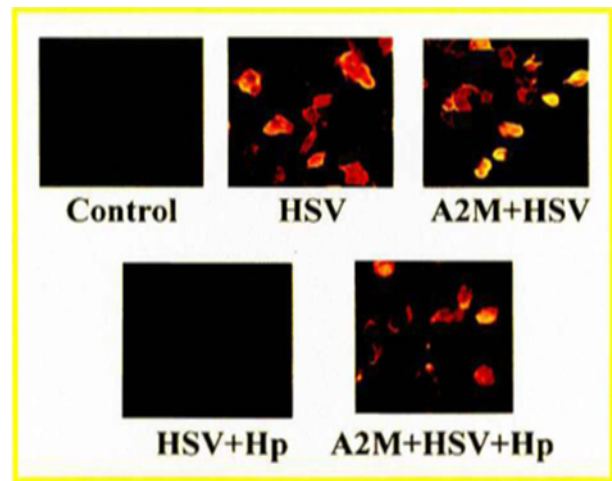
(Table 1). This effect was not the result of A2M cytotoxicity, because the synthesis of cellular proteins was not altered when compared to controls and treatment with A2M alone did no affect cellular viability (data not shown).

To ascribe the experimental results to the induction of a restrictive mechanism, we further analyzed the molecular effect of A2M on HSV-1-infected neuroblastoma cells. First, we considered the possibility that HSV-1 induced the production of NO by SKNMC, because previous findings from our laboratory showed that HSV-1 induced the production of this gas by other cell lines (López-Guerrero and Alonso, 1997). Figure 4 shows that, indeed, HSV-1 activates the production of NO, although this induction was clearly significant only when large amounts of infectious particles were added. Curiously, A2M increased the accumulation of NO in

Table 1 Effect of A2M on the susceptibility of SKNMC to HSV-1 infection

Pretreatment	Virus production (PFU/cell)*	Cell viability**
None	40	<5
A2M (150 mg/ml)	9	<5
Heparin (10 mg/ml)	0.3	>95
Heparin + A2M	0.6	73

*Virus titrations were performed by plaque assay on Vero cell monolayers.
**Survival of HSV-1-infected cells was determined by the trypan blue exclusion technique and the values obtained are expressed as a percentage relative to the survival of mock-infected cultures. Values are means from three independent experiments (SD < 22%).
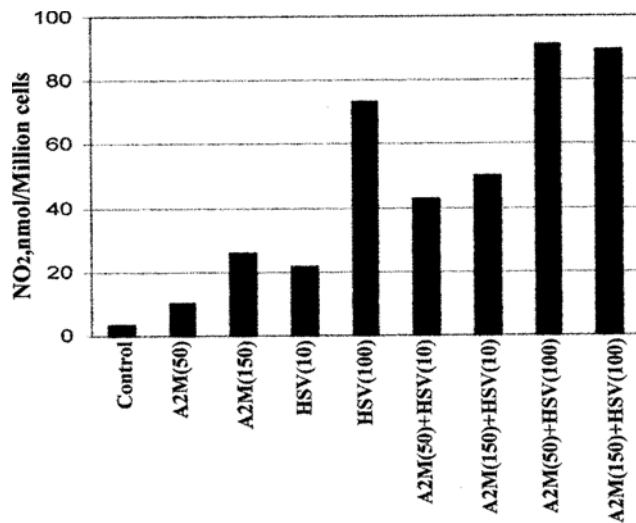
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Figure 4 Induction of NO production in SKNMC. Cells, pretreated or not with A2M (50 or 150 µg/ml), were mock-infected or infected with HSV-1 at the indicated PFU per cell. At 24 h p.i., the accumulation of NO was assayed. Values are means of three experiments, performed in triplicate.

all HSV-1 infected culture (Figure 4). Nevertheless, the sole treatment with the protease inhibitor produced small amounts of NO, and addition of the NOS inhibitor L-NMMA was unable to modify the course of the infection (data not shown), suggesting that the effect of A2M might not be fundamentally directed through the activation of NOS enzymes.

Finally, we investigated whether the effect of A2M on HSV-1 infected SKNMC could be due to apoptosis induction. For this purpose, internucleosomal DNA fragmentation and nuclear fluorescence staining was analyzed in A2M treated cells infected or not with HSV-1 at 24 h p.i. (Figure 5). Under these conditions, A2M-treated cultures produced DNA degradation and chromatin condensation, comparable to those obtained after treatment with cycloheximide, a known inducer of apoptosis (López-Guerrero *et al*, 2000). The appearance of apoptotic features seemed to increase in A2M-pretreated HSV-1-infected cultures. This result suggests that the restrictive infection observed in A2M-treated cultures might be due to induction of apoptosis, which could hinder the correct assembly and release of virions.
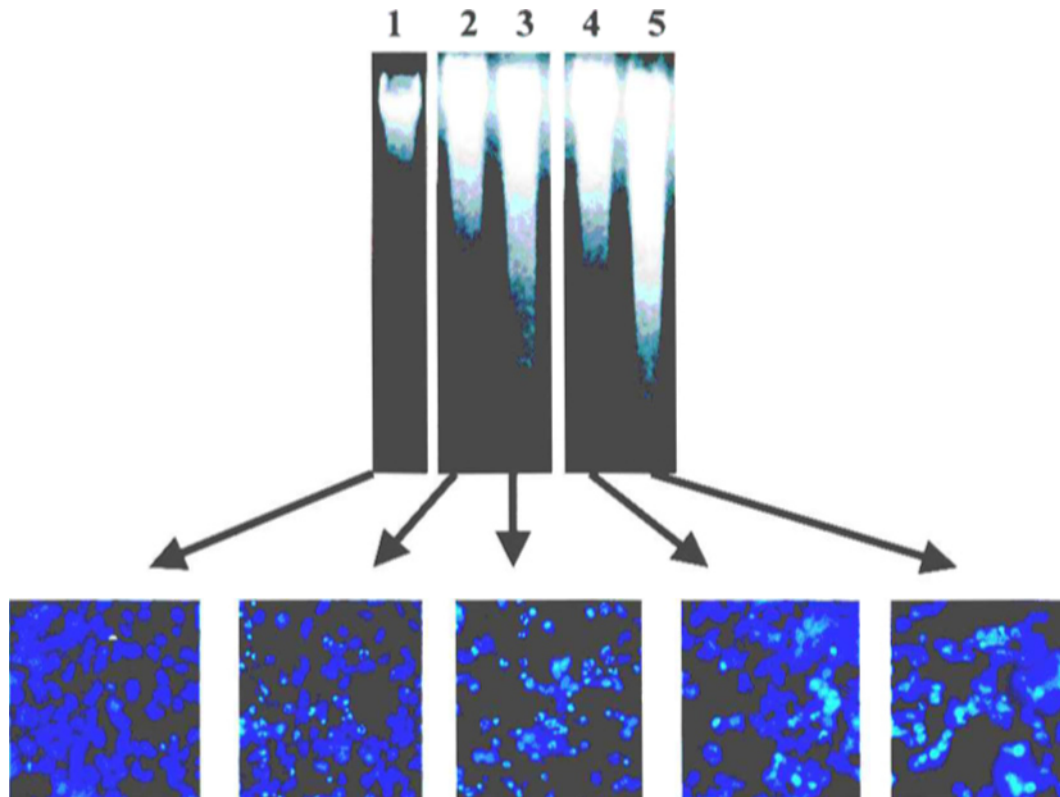

Figure 5 DNA fragmentation and nuclear fluorescence analysis in HSV-1-infected SKNMC treated with A2M. One million cells were treated or not with A2M and/or infected or not with HSV-1. At 24 h p.i., cells were collected by low-speed centrifugation and processed for DNA analysis as described in the Materials and methods. Control (**1**); treated with 150 µg/ml A2M (**2**); treated with cycloheximide for 24 h at 10 µg/ml as a control of apoptosis induction (**3**); HSV-1 infection at 10 PFU per cell (**4**); pretreated with A2M and infected with HSV-1 at 10 PFU per cell (**5**). Fluorescence microscopy analysis of cells stained with DAPI solution was carried out in parallel.

Discussion

A2M is a 720-kDa protein consisting of four identical subunits of 180 kDa, responsible for the binding and inactivation of plasma proteases and transport of various types of molecules (Borth, 1992; Krimbou *et al*, 1998). In the present report, we show that A2M binds HSV-1 particles *in vitro*. Although it seems to be very unlikely that A2M could bind the HSV-1 protease VP24, a very recent publication points out that unlike VP21 and VP22a, which are removed from mature capsids, VP24, which contains the protease domain, could be present in all types of HSV-1 capsids (Sheaffer *et al*, 2000). Whether this presence is sufficient to interact with A2M remains to be resolved.

In addition to the capacity of A2M to bind a broad range of molecules, there are many aspects to be considered to support the possibility of an interaction between this protease inhibitor and HSV-1 *in vivo*. On the one hand, both elements can be present and internalized into neurons of the CNS (Ganter *et al*, 1991; Immergluck *et al*, 1998). Furthermore, it is a matter of speculation whether A2M facilitates internalization of HSV through the low-density lipoprotein receptor-related protein (LRP), a heterodimer glycoprotein and receptor of A2M (Moestrup, 1994). On the other hand, it is worth noting that both A2M and HSV-1 particles have been associated with apoE (Corder *et al*, 1998; Krimbou *et al*, 1998), which uses A2M/LRP and LRP-related receptors to reach astrocytes and neurons (Borth, 1992; Moestrup, 1994). Moreover, an increasing number of studies directly or indirectly implicate all these elements in important neurodegenerative pathologies, such as Alzheimer's disease (Strauss *et al*, 1992; Itabashi *et al*, 1997; Blacker *et al*, 1998; Bullido *et al*, 1998; Corder *et al*, 1998; Krimbou *et al*, 1998; Leissring *et al*, 1998; Dow *et al*, 1999; Rudrasingham *et al*, 1999; Singleton *et al*, 1999). In addition, a recent report describes an HSV-1 glycoprotein B fragment homologous to the Alzheimer's Aβ peptide, which could be involved in fibril formation and neurotoxicity (Cribbs *et al*, 2000). Thus, although the present study provides evidence of *in vitro* binding, a more biological interaction in the CNS between A2M and HSV-1 may be implied.

Despite the fact that the physiological significance of these interactions remains to be determined, it has been observed that: a) A2M interferes with the onset of herpes viral keratitis (Chesnokova and Maichuk, 1986); b) Association of apoE with A2M and HSV-1 is dependent on apoE phenotype (Krimbou *et al*, 1998; Leissring *et al*, 1998; Corder *et al*, 1998; Itzhaki *et al*, 1997); and c) Association of apoE, HSV-1 and A2M with neurodegeneration depends, likewise, on the polymorphism of both apoE and A2M molecules (Itzhaki *et al*, 1997; Blacker *et al*, 1998; Bullido *et al*, 1998; Corder *et al*, 1998; Leissring *et al*, 1998; Dow *et al*, 1999; Rudrasingham *et al*, 1999; Singleton *et al*, 1999); and d) HSV-1 infection in the CNS has been associated with the pro-inflammatory IL-6 (Carr and Campbell, 1999; LeBlanc *et al*, 1999), which can strongly induce A2M synthesis, suggesting that human A2M behaves as an acute-phase protein, leading to the onset of neurodegenerative manifestations (Strauss *et al*, 1992).

We have observed the exacerbation of the CPE induced after treatment with A2M in HSV-1 infected neuroblastoma cells. This cellular death did not correlate with viral particle production, suggesting that, despite the possible increased synthesis of viral proteins, pretreatment with the protease inhibitor could induce restrictive infection. Whether the binding of HSV-1 induces conformational changes and activation of A2M remain to be determined, although it seems very likely that it will depend on the nature of the bound target, because only association of proteolytic proteins seems to cause a significant conformational change in A2M (Krimbou *et al*, 1998). The fact that A2M is not a heparin-binding protein (Soker *et al*, 1993) reduces the possibility that the reversion of the effect exerted by heparin in HSV-1-infected SKNMC cells could be due to the direct interaction of A2M with heparin, which would favor virus uptake. Whether direct interaction between A2M and HSV is necessary to aggravate the CPE will be, likewise, further studied.

Experiments carried out in our laboratory demonstrated that HSV-1 induces the accumulation of the gas NO (López-Guerrero and Alonso, 1997). Likewise, HSV-1 activated the NOS enzyme in the SKNMC neuroblastoma cell line. It is of note that A2M increased the detection of NO accumulation after viral infection. This seemed to be very significant, given that this gas is a pleiotropic effector in the CNS, which is known to be involved in cerebral vasculopathy and neurodegeneration (de la Monte *et al*, 2000). In addition, NOS activity inhibits HSV-1 replication in neurons (Komatsu *et al*, 1996). Nevertheless, as in previous studies (López-Guerrero and Alonso, 1997; López-Guerrero and Carrasco, 1998; Fujioka *et al*, 1999), inhibition of the production of NO did not modulate either the infection or the action of A2M. This and the fact that HSV-1 alone, but not A2M, induced production of NO suggest that the effect of pretreatment with A2M is probably exerted through an alternative pathway and the role of the accumulation of NO remains unexplained.

Preliminary results suggest that some characteristics of apoptosis arise in SKNMC infected with HSV-1 and pretreated with A2M. Although wild type HSV-1 has been mainly involved in apoptosis inhibition (Galvan *et al*, 1999; Koyama and Miwa, 1997; Laxminarayana *et al*, 1999) and only some virus mutants trigger programmed cell death (Galvan and Roizman, 1988; Galvan *et al*, 1999, 2000), in our system, HSV-1 infection seemed not to block A2M-induced apoptosis. Pretreatment with A2M produces DNA degradation and chromatin condensation in SKNMC. Given that a previous study showed

that HSV-1 blocks caspase-independent and caspase-dependent pathways to apoptosis (Galvan *et al*, 1999), the mechanism of A2M-induced apoptosis and the implication or not of caspases requires further investigation.

In conclusion, this study provides the first evidence of A2M and HSV-1 interaction *in vitro* and in infected neuroblastoma cells, induction of NO production in infected cells, pretreated or not with A2M and, maybe, the subsequent NO-independent induction of programmed cell death. The precise implications, if any, of this interaction in the development of some neuropathologies affecting the CNS remain unresolved.

Materials and Methods

Cells and virus
The human neuroblastoma SKNMC cell line was cultured in MEM (Life Technologies, Paisley, United Kingdom) supplemented with 10% heat-inactivated fetal calf serum (FCS) and nonessential amino acids in a 5% CO_2 atmosphere at 37°C. HSV-1 (Kos strain) was propagated in Vero cells and purified through a 30% sucrose cushion. Virus titration by plaque assay was performed on Vero cell monolayers with a final concentration of 0.5% agar (Gibco BRL, Life Technologies). The m.o.i. was expressed as the number of PFU per cell. Experiments were carried out with exponentially growing cultures.

ELISA
Poly-(vinyl chloride) 96-well plates (Costar) were coated with 50 μl of A2M (Calbiochem, San Diego, CA, U.S.A.) at 10 or 0.01 ng/ml by overnight incubation at 37°C. Non-specific binding sites were blocked with PBS containing 3% (w/v) BSA at 37°C for 30 min. Subsequently, 10 μl of HSV-1 was added directly (3×10^8 PFU/ml) or previously diluted (1:10) with PBS at 37°C for 30 min. Nonbound HSV-1 was removed by washing 3 times with PBST (PBS + 0.05% Tween 20). After incubation with anti-HSV-1 polyclonal antibodies (Dako, Glostrup, Denmark) in PBST-BSA (PBST + 1% (w/v) BSA) at room temperature for 30 min, plates were washed with PBST (rinsed 3 times) and incubated in the same conditions with horseradish peroxidase-coupled goat anti-rabbit immunoglobulins (Dako). Bound peroxidase was detected with *o*-phenylene-diamine in the presence of hydrogen peroxide. Absorbance at 490 nm was determined in a microplate reader (Dynatech, Billingshurst, West Sussex, United Kingdom).

Immunoprecipitation and western blotting
Experiments of immunoprecipitation were performed *in vitro* basically as described by Romero *et al* (1999) Briefly, 6×10^6 PFU of HSV-1 were incubated with 10 μg A2M for 1 h at 37°C. Subsequently, the mixture was resuspended in PBS and immunoprecipitated with 1:100 dilution of a polyclonal anti-A2M antibody (Biomeda Corp, Foster City, CA, U.S.A.) or irrelevant antibody. Protein A-agarose was added for 1 h at 4°C and, after washing, immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose and reciprocal Western blotting was performed with anti-A2M or an anti-HSV-1 (Dako) polyclonal antibodies.

Immunofluorescence analysis
Cultures were pretreated or not with heparin (Sigma) at 100 μg/ml or A2M (150 μg/ml) and mock-infected or infected with HSV-1 (1 PFU per cell). Then, 24 h p.i., cells were fixed with acetone at -20°C for 10 min and washed with PBS. A 1:100 dilution of polyclonal anti-HSV-1 antibody was added and incubated for 1 h at 37°C temperature. Cells were washed thoroughly with PBST and a rhodamine-labeled goat anti-rabbit IgG antibody (PIERCE, Illinois, USA) was added at 1:200 dilution. After 30 min at 37°C, cells were washed and examined using a Nikon fluorescence microscope.

NO production
The production of NO was measured as described by Green *et al*, (1982). In each individual sample, aliquots of SKNMC supernatants (0.1 ml), pretreated or not with A2M and uninfected or infected with HSV-1, were incubated, in triplicate, in flat-bottomed 96-well culture plates and mixed with the same amount of Greiss reagent [0.1% naphthyl-ethylenediamine dihydrochloride (Sigma) in distilled water and 1% sulfanilamide (Sigma) in 5% phosphoric acid (vol/vol)]. Subsequently, this mixture was incubated for 10 min at room temperature and the optical density at 550 nm was measured in a MR 5000 microplate reader (Dynatech).

DNA fragmentation and nuclear fluorescence analysis
Detection of internucleosomal DNA fragmentation was measured by electrophoresis in a 2% agarose gel as previously described (López-Guerrero *et al*, 2000). Briefly, one million cells were resuspended with 1:1 sample buffer (10% glycerol, 10 mM Tris, pH 8, 0.1% [w/v] bromophenol blue) and RNase A [10 mg/ml] and loaded directly onto the agarose gel, where the section immediately above the comb was removed and filled with 1% agarose, 2% SDS, and 64 μg/ml protease K in TBE buffer. After electrophoresis, the gel was stained with 2 μg/ml ethidium bromide. Nucleus DAPI (4′,6-Diamidino-2-Phenylindole; Sigma) staining was performed following standards protocols.

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