Suppression of HIV-1 Infectivity by Human Glioma Cells

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Abstract

HIV-1 infection to the central nervous system (CNS) is very common in AIDS patients. The predominant cell types infected in the brain are monocytes and macrophages, which are surrounded by several HIV-1-resistant cell types, such as astrocytes, oligodendrocytes, neurons, and microvascular cells. The effect of these HIV-1-resistant cells on HIV-1 infection is largely unknown. In this study, we examined the stability of HIV-1 cultured with several human glioblastoma cell lines, for example, NP-2, U87MG, T98G, and A172, to determine whether these HIV-1-resistant brain cells could enhance or suppress HIV-1 infection and thus modulate HIV-1 infection in the CNS. The HIV-1 titer was determined using the MAGIC-5A indicator cell line as well as naturally occurring CD4⁺ T cells. We found that the stability of HIV-1 incubated with NP-2 or U87MG cells at 37°C was significantly shorter (half-life, 2.5–4 h) compared to that of HIV-1 incubated with T98G or A172 cells or in culture medium without cells (half-life, 8–18 h). The spent culture media (SCM) of NP-2 and U87MG cells had the ability to suppress both R5- and X4-HIV-1 infection by inhibiting HIV-1 attachment to target cells. This inhibitory effect was eliminated by the treatment of the SCM with chondroitinase ABC but not heparinase, suggesting that the inhibitory factor(s) secreted by NP-2 and U87MG cells was chiefly mediated by chondroitin sulfate (CS) or CS-like moiety. Thus, this study reveals that some but not all glioma cells secrete inhibitory molecules to HIV-1 infection that may contribute in lowering HIV-1 infection in the CNS *in vivo*.

Introduction

THE HUMAN BODY is composed of trillions of cells that encompass at least 400 types.^{1,2} Among these, only a few cell types that express both CD4 and an appropriate coreceptor, mainly CCR5 for CCR5-tropic HIV-1 (R5-HIV-1) or CXCR4 for CXCR4-tropic HIV-1 (X4-HIV-1), are susceptible to HIV-1 infection, such as CD4⁺ T cells, dendritic cells, and macrophages.^{3,4} HIV-1 crosses mucosal barriers by infecting dendritic cells and macrophages.^{5–7} These cells then convey the infection to lymphatic tissues in the lymph nodes, spleen, and gut, where CD4⁺ T cells are the prime targets for infection.⁸ At later stages, HIV-1 is disseminated to many important organ systems, including the central nervous system (CNS), lung, liver, kidney, and adrenal and other endocrine organs.^{8,9} However, throughout this long course of infection, HIV-1 infection mainly remains restricted within HIV-1-susceptible CD4⁺ T cells, dendritic

cells, and macrophages, although a few other cell types, such as chromaffin cells,^{10,11} astrocytes,^{12,13} and neurons,^{14,15} are often found to be infected with HIV-1. Thus, the majority of cells in the body remain resistant to HIV-1 infection, and the effects of these cells on HIV-1 infection have been poorly investigated. Typically, HIV-1 infection and its production *in vivo* occur at much lower levels compared to *in vitro* systems,⁸ but the mechanism underlying this phenomenon is not fully understood.

HIV-1 infection to the CNS causes neurological abnormalities, such as HIV encephalopathy, peripheral neuropathy, and the AIDS dementia complex, which are very common in AIDS patients.^{16,17} In the brain, HIV-1 mainly infects microglia, monocytes, and macrophages, which are surrounded by various HIV-1-resistant cell types, such as astrocytes, oligodendrocytes, neurons, and microvascular cells.^{18,19} Previously, we showed that these HIV-1-resistant cells could be incorporated into syncytia (multinucleated

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giant cells) by fusion with HIV-1-infected cells, resulting in healthy bystander cell death.²⁰ However, whether these HIV-1-resistant cells play any role in the enhancement or suppression of HIV-1 infection remains unknown. For instance, dendritic cells, expressing the C-type lectin DC-SIGN, have been shown to retain viral infectivity,^{21–23} whereas CD8⁺ T cells, for example, the TG-cell line, have been reported to suppress HIV-1 transcription by exosome secretion.^{24–26}

In this study, we investigated the stability of HIV-1 following incubation with human glioma cell lines to evaluate whether human glioma cells had any effect on HIV-1 infection. We found that the NP-2 and U87MG human glioma cell lines secreted macromolecules that suppressed HIV-1 infection. Finally, we investigated the underlying mechanism.

Materials and Methods

Cells

The human T-cell line C8166²⁷ and its derivative C8166/CCR5 (CCR5-transduced C8166 cells),²⁷ and MOLT4/IIIB²⁸ cells (MOLT4 human T cells persistently infected by HIV-1 IIIB), were cultured in RPMI 1640 medium (Nissui Co. Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), designated 10% RPMI.

We used four human glioblastoma cell lines, NP-2,^{20,29} U87MG,³⁰ A172,³¹ and T98G,^{31,32} and the HeLa³³ cervical carcinoma cell line in this study. These cells were maintained in Eagle's minimum essential medium (Nissui Co., Ltd.) with 10% FBS (10% EMEM).

MAGIC-5A cells, clone 1–10,^{34,35} were MAGI cells (HeLa/CD4/LTR- β -gal) transduced to express CCR5 and were maintained in 10% EMEM containing 1 μ g/ml of blasticidin-s (Calbiochem, San Diego, CA).

HIV-1 strains

The CCR5-tropic HIV-1 (R5-HIV-1) strains, Ba-L, SF162, and 92US723, were propagated in C8166/CCR5 cells. The CXCR4-tropic HIV-1 (X4-HIV-1) strain IIIB was produced by MOLT4/IIIB cells. Two other X4-HIV-1 strains, 4803 and 94UG103, were propagated in C8166 cells.

Preparation of spent culture media

Glioblastoma or HeLa cells were seeded (12×10^4 cells/ml) into cell culture dishes and incubated at 37°C in the presence of 5% CO₂ for 48 h. Spent culture media (SCM), that is, culture supernatants on cells, were collected, centrifuged at 1,000 rpm for 5 min, and stored in aliquots at –80°C before use. Culture medium alone, that is, 10% EMEM without cells, was similarly incubated, processed, and stored at –80°C until use.

Detection of HIV-1 infectivity

HIV-1 infectivity was determined mainly using MAGIC-5A indicator cells, clone 1–10, as described previously.³⁴ MAGIC-5A cells are HeLa cells that endogenously express CXCR4 and are transduced to express CD4 and CCR5 on the cell surface. As a result, these cells are susceptible to both X4- and R5-HIV-1. As these cells express β -galactosidase (β -gal) under the control of an HIV-1 LTR promoter, deep blue HIV-1-infected cells can be detected under a light microscope after X-gal staining.

MAGIC-5A cells were seeded (2.5×10^4 cells in 250 μ l/well) onto 48-well plates. The next day, the medium was removed; 135 μ l of SCM and 15 μ l of HIV-1 inoculum were added per well sequentially and incubated for 2–3 h. Then, the cells were washed once with 10% EMEM and incubated for further 2 days. HIV-1 infection was detected on day 2 after staining for X-gal, as described previously.^{34,36}

To determine HIV-1 infectivity using C8166/CCR5 cells, 1×10^5 C8166/CCR5 cells were suspended in 180 μ l of SCM in Eppendorf tubes, followed by inoculation with 20 μ l of HIV-1. The cells were then incubated in the tubes for 2–3 h at 37°C with 5% CO₂, washed once with 10% RPMI, and plated on 48-well plates. After incubation for 2 days, HIV-1-infected cells were detected using the indirect immunofluorescence assay (IFA), as described previously.²⁰

Stability of HIV-1 incubated with different cell lines

HIV-1 was incubated with NP-2, U87MG, A172, T98G, and HeLa cells for 24, 12, 6, 3, or 0 h, and remaining HIV-1 titers were determined as follows. These cells were seeded (3×10^4 cells in 250 μ l/well) onto 48-well plates and incubated overnight. The medium was removed, and the cells were inoculated with HIV-1 (150 μ l/well) according to the planned intervals so that HIV-1 could be incubated with each cell line for 24, 12, 6, 3, or 0 h. Each experiment was conducted with HIV-1 aliquots of the same lot stored at –80°C. Immediately after the last inoculation (i.e., at 0 h), 50 μ l of the culture supernatants were collected from all wells, and the infectivity of the HIV-1 was determined using MAGIC-5A cells.

Statistical analysis

Pearson correlation coefficients (r) with p -values and differences between the means of the experimental groups were determined using the two-tailed t -test by MedCalc, version 9 (MedCalc Software, Mariakerke, Belgium).

Results

Stability of HIV-1 incubated with glioblastoma cell lines

R5-HIV-1 Ba-L was incubated with four glioblastoma cell lines, NP-2, U87MG, A172, and T98G, at 37°C. As controls, HIV-1 was similarly incubated with a nonglioblastoma cell line, HeLa, and in culture medium alone (10% EMEM without cells). After incubation for different periods (24, 12, 6, 3, or 0 h), culture supernatants containing HIV-1 were collected, and the HIV-1 titers were determined using MAGIC-5A indicator cells.

As shown in Figure 1a, the HIV-1 titers decreased at each time point under all experimental conditions. The HIV-1 titers decreased significantly faster when the virus was incubated with NP-2 or U87MG cells compared to the A172, T98G, HeLa cells, or without cells in culture medium alone (Fig. 1a). The half-lives of HIV-1 Ba-L incubated with NP-2 or U87MG cells were 2.5 to 4 h, while those of HIV-1 Ba-L incubated with A172, T98G, HeLa cells, or without cells in culture medium alone were 8 to 18 h (Fig. 1b). Thus, incubation of HIV-1 with A172 or T98G cells barely affected viral stability.

The rapid decrease in HIV-1 titers following incubation with the NP-2 and U87MG cells raised two possibilities:

FIG. 1. Stability of HIV-1 infectivity incubated with various glioblastoma cell lines. **(a)** Infectivity of HIV-1 Ba-L following incubation with various glioblastoma cell lines. HIV-1 Ba-L was incubated with various glioblastoma cell lines or without cells in culture medium alone for different time intervals, as described in the Materials and Methods section. Virus supernatant was then collected and used to infect MAGIC-5A indicator cells. The numbers of infected cells indicating viral titers following incubation with different glioblastoma cell lines for indicated time intervals are shown. HIV-1 titers decreased with time in all conditions but significantly faster while incubation with NP-2 and U87MG cells. **(b)** The half-lives of HIV-1 infectivity following incubation with various cell lines are shown. All data are the means $\pm$ SD of three independent experiments performed in duplicate. *Significantly ($p < .05$) low values.

(1) more HIV-1 Ba-L attached to these cells and/or (2) the cells secreted molecules capable to suppress HIV-1 infectivity.

To examine the first possibility, we determined the amount of HIV-1 bound to the cells, as described elsewhere.²⁸ Namely, NP-2 and HeLa cells were inoculated with HIV-1 Ba-L and incubated overnight at 37°C. The cells were then washed once with 10% EMEM and subjected to an enzyme-linked immunosorbent assay (ELISA) for HIV-1 p24. HIV-1 binding to the NP-2 and HeLa cells was similar (Fig. 2a), suggesting that there was little difference between the amounts of HIV-1 Ba-L present in the culture media of the NP-2 and HeLa cells.

Next, to examine the second possibility and rule out the first possibility, we investigated whether the cells secreted molecules that suppressed HIV-1 infectivity. For this, we examined the infectivity of HIV-1 mixed with SCM from these cell lines.

Examination of the infectivity of HIV-1 mixed with SCM from glioblastoma cells

To prepare the SCM, cells (NP-2, U87MG, A172, T98G, and HeLa) were cultured in 10% EMEM for 48 h. The SCM were collected, centrifuged, and stored at -80°C before use. We examined the effects of SCM on the infectivity of HIV-1

Ba-L in MAGIC-5A indicator cells. Namely, SCM were overlaid onto preseeded MAGIC-5A cells. The cells were then inoculated with HIV-1 Ba-L for 2–3 h, washed once with culture medium, and incubated for further 2 days.

SCM from NP-2 and U87MG cells but not A172, T98G, or HeLa cells significantly ($p < .01$) suppressed HIV-1 infectivity compared to culture medium alone (Fig. 2b). It suggests that the NP-2 and U87MG cells secrete molecules that affect the infectivity of HIV-1 Ba-L. Here, HIV-1 was not preincubated with SCM to inhibit the infection, indicating that SCM of day 2 were effective to inhibit HIV-1 infection to target cells.

Suppression of the infectivity of different HIV-1 strains by NP-2 SCM

Next, we examined whether the SCM from NP-2 cells could suppress the infectivity of other R5- or X4-HIV-1 strains. For this, we examined the infectivity of other R5-HIV-1 (SF162 and 92US723) and X4-HIV-1 (IIIB, 4803, and 94UG103) strains in the presence of NP-2 SCM or culture medium alone using MAGIC-5A indicator cells. Most strains exhibited $<50\%$ infectivity in the presence of NP-2 SCM compared to culture medium alone (Table 1). These data reveal that NP-2 SCM can inhibit infection of both R5- and X4-HIV-1 strains.

FIG. 2. (a) HIV-1 Ba-L bindings to cell lines. NP-2 and HeLa cells were incubated with or without HIV-1 Ba-L overnight at 37°C. Cells were then washed once with 10% EMEM and subjected to enzyme-linked immunosorbent assay (ELISA) for HIV-1 p24. Optical densities (OD) at 450 nm indicating binding HIV-1 p24 level in cells have been shown. Both NP-2 and HeLa cells showed similar levels of HIV-1 bindings. (b) Suppression of HIV-1 infectivity by spent culture media (SCM) from various glioblastoma cell lines. The SCM from different cell lines (T98G, A172, U87MG, NP-2, and HeLa) and culture medium alone were overlaid onto preseeded MAGIC-5A indicator cells and infected with HIV-1 Ba-L. The relative percentages of infected cells are shown. The SCM of NP-2 and U87MG cells inhibited HIV-1 infectivity. *Significantly ($p < .05$) low values.

To confirm this result using a naturally occurring CD4⁺ T-cell line, SCM from NP-2 or HeLa cells or culture medium alone were mixed with R5-HIV-1 Ba-L or X4-HIV-1 IIIB and allowed to infect CCR5-transduced C8166 human T cells (C8166/CCR5) for 2–3 h. Infected cells were detected by the

TABLE 1. SUPPRESSION OF THE INFECTIVITY OF DIFFERENT HIV-1 STRAINS BY NP-2 SPENT CULTURE MEDIA

HIV-1	Strains	Infectivity		
		Media alone	NP-2 SCM	% Inhibition
R5	Ba-L	50 ± 15 ^a	20 ± 1	60
	SF162	132 ± 70	44 ± 28	66
	92US723	36 ± 14	16 ± 5	55
X4	IIIB	58 ± 2	31 ± 2	46
	4803	74 ± 1	28 ± 4	62
	94UG103	87 ± 1	42 ± 5	52

^aNP-2 SCM or culture media alone were overlaid onto preseeded MAGIC-5A indicator cells and infected with HIV-1 for 2 h. Cells were then washed once with fresh culture media (10% EMEM) and incubated for further 2 days. HIV-1-infected cells were detected after X-gal staining. The X-gal-positive cells per well ± SD of three independent experiments performed in duplicate are shown.

SCM, spent culture media.

indirect IFA on day 2 postinfection. The SCM from NP-2 cells significantly suppressed the infectivity of both the R5 Ba-L ($p = .04$) and X4 IIIB ($p = .01$) strains compared to the HIV-1 strains incubated with HeLa SCM or culture medium alone (Fig. 3a). Thus, the suppression of both R5- and X4-HIV-1 infection by NP-2 SCM was confirmed using established cells derived from naturally occurring HIV-1-susceptible CD4⁺ T cells.

Effect of NP-2 SCM collected on different days on HIV-1 infectivity

We collected NP-2 SCM on days 1–3 and compared its anti-HIV-1 activity with the SCM similarly collected from HeLa cells or culture medium alone. HIV-1 infectivity was significantly ($p < .05$) reduced even in the presence of NP-2 SCM of day 1, and the inhibitory activity was increased with SCM of days 2 and 3. In contrast, the infectivity of HIV-1 following incubation with HeLa SCM or culture medium alone was similar, which did not alter for using SCM of different days (Fig. 3b). A very highly significant and a strong negative correlation ($r = -0.98$; $p = .001$) existed between the anti-HIV-1 activity and the volume of NP-2 SCM (Fig. 3c).

Inhibitory factor(s) eliminated by filtration but not by heating

To characterize the inhibitory factor(s) present in the SCM, the SCM of NP-2 and HeLa cells and culture medium alone were heated at 100°C for 30 min or filtered through 100 K filters by centrifugation at 2,000×g for 25 min. HIV-1 Ba-L was allowed to infect MAGIC-5A indicator cells for 2 h in the presence of these treated SCM. The cells were then washed once with fresh medium, and the HIV-1 infectivity was examined after incubation for 2 days with fresh medium. The anti-HIV-1 activity of the NP-2 SCM was abolished by filtration but not by heating at 100°C (Fig. 4a), suggesting that the SCM of NP-2 cells contain inhibitory molecules that are heat resistant and have molecular weights >100 KDa.

Inhibition of HIV-1 binding to target cells by the NP-2 SCM

To elucidate the specific step of the HIV-1 life cycle influenced by the NP-2 SCM, we examined whether HIV-1 binding was affected by NP-2 SCM. To this end, preseeded MAGIC-5A cells were incubated with the R5 Ba-L virus for 2–3 h in the presence of SCM from NP-2 or HeLa cells or culture medium alone. The cells were then washed several times with PBS to remove free viruses and subjected to an ELISA for HIV-1 p24. Approximately 50% of the HIV-1 binding was inhibited in the presence of the NP-2 SCM, whereas HIV-1 binding in the presence of the HeLa SCM was similar to that observed with culture medium alone (Fig. 4b).

To examine whether the NP-2 SCM had any effect on the postentry steps of HIV-1 replication, MAGIC-5A cells were infected with HIV-1 Ba-L at 37°C for 2 h. The cells were then washed and incubated for 2 days with the NP-2 SCM or culture medium alone. HIV-1-infected cells were detected by X-gal staining. Similar HIV-1 infections were detected for incubation with NP-2 SCM or culture medium alone, suggesting that NP-2 SCM barely affected the postentry steps of HIV-1 replication (Fig. 4c).

FIG. 3. Suppression of HIV-1 infectivity by NP-2 SCM. **(a)** Inhibition of HIV-1 infection to human T cells. C8166/CCR5 cells were infected with the R5 Ba-L or X4 IIIB strains in the presence of culture medium alone (10% EMEM), HeLa SCM, or NP-2 SCM for 2 h at 37°C in Eppendorf tubes. Cells were then washed once with fresh media, plated on a 48-well plate, and incubated for 2 days in fresh media. HIV-1 antigen-positive cells were detected by indirect immunofluorescence assay. The relative percentages of HIV-1 antigen-positive cells are shown. The presence of the NP-2 SCM but not the HeLa SCM significantly inhibited the infection of both R5- and X4-HIV-1 to C8166/CCR5 cells. **(b)** Effect of SCM collected on different days on HIV-1 infectivity. NP-2 cells, HeLa cells, and culture medium alone were incubated for 1–3 days, and the SCM were collected on each day. These SCM (135 μ l) were mixed with HIV-1 Ba-L (15 μ l) and used to infect MAGIC-5A cells. The number of HIV-1-infected cells (X-gal stained) per well is shown. Even the NP-2 SCM collected on day 1 significantly inhibited HIV-1 infectivity. **(c)** Association between HIV-1 infectivity and volume of NP-2 SCM. Different volumes of NP-2 SCM were mixed with fresh media (10% EMEM) and overlaid (135 μ l) onto preseeded MAGIC-5A cells. The cells were then infected with HIV-1 Ba-L (15 μ l) for 2 h, washed, and incubated for 2 days in fresh media. HIV-1-infected cells were detected by X-gal staining. Numbers of infected cells are presented in relation to the volume of NP-2 SCM used. The calculated correlation coefficient (r) and p -value are shown. All data are the means $\pm$ SD of three independent experiments performed in duplicate. *Significantly ($p < .05$) low values.

In contrast, when MAGIC-5A cells were infected with HIV-1 Ba-L in the presence of NP-2 SCM or culture medium alone at 37°C for 2 h, washed once, and incubated in fresh medium for 2 days, the titers of HIV-1 inoculated in the presence of the NP-2 SCM were significantly ($p = .0004$) reduced compared to those of HIV-1 inoculated in culture medium alone (Fig. 4c). Thus, the presence of NP-2 SCM during the binding stage but not at later stages significantly inhibited HIV-1 infectivity.

Collectively, our data reveal that NP-2 cells secrete inhibitory molecules that inhibit HIV-1 binding to target cells, and these molecules have little effect on the postentry steps of the HIV-1 life cycle.

Treatment of SCM with chondroitinase ABC but not heparinase eliminated the inhibitory effect

Previous studies have shown that many cell lines,³⁷ such as human B-cell lines,³⁸ and normal and malignant breast cell lines³⁹ secrete proteoglycans, including chondroitin sulfate

(CS) and heparan sulfate. Some of these proteoglycans have been reported to inhibit the infection of retroviruses, including HIV-1 and HTLV-1.^{40–42} Therefore, we examined the concentrations of sulfated glycosaminoglycans (sGAG) in the SCM using sGAG quantitative kit from Wieslab (Euro-Diagnostica AB, Medeon, Sweden). The sGAG content in the SCM of both the NP-2 and U87MG cells was higher than that in the SCM of the HeLa, T98G, A172 cells, and culture medium (data not shown). Previously, Newburg *et al.*⁴³ have shown that human milk contains glycosaminoglycans that inhibit HIV-1 binding to target cells, and this activity was lost when milk was treated with a lytic enzyme specific for CS but not enzymes specific for heparin, heparan sulfate, or dermatan sulfate. Therefore, we planned to treat SCM with lytic enzymes specific for different glycosaminoglycans.

NP-2 SCM and culture medium alone were mixed with either chondroitinase ABC or heparinase (EC-4.2.2.7) and incubated at 37°C for 30 min. After inactivating the enzymes by heating at 65°C for 10 min, the SCM were cooled on ice, mixed with HIV-1 Ba-L, and used to infect MAGIC-5A cells.

FIG. 4. Characterization of the inhibitory factor(s) and detection of the underlying mechanism. (a) Elimination of the inhibitory component by filtration. The NP-2 SCM, HeLa SCM, and culture medium alone were heated at 100°C for 30 min or filtered through 100 K filter at 2,000×g for 25 min. Treated and nontreated culture supernatants were mixed with HIV-1 Ba-L, and infectivity was examined in MAGIC-5A cells. Significant inhibition existed after heating but not after filtration. (b) Inhibition of HIV-1 binding to target cells. MAGIC-5A cells were incubated with the R5 Ba-L virus for 2–3 h in the presence of NP-2 SCM, HeLa SCM, or culture medium alone and washed several times with PBS. The cell lysates were subjected to ELISA for HIV-1 p24 detection. The relative percentages of HIV-1 p24 in the cell lysates are shown. (c) Less effect was observed on the postentry steps of HIV-1 replication. When MAGIC-5A cells were infected in the presence of NP-2 SCM or culture medium alone (CM), followed by washing and incubation for 2 days in fresh culture media, inhibition of HIV-1 infectivity was observed. However, when MAGIC-5A cells were infected with HIV-1 Ba-L in the presence of fresh media, followed by washing and incubation of the cells for an additional 2 days in the presence of NP-2 SCM or CM, no inhibition of HIV-1 infection was observed. Data are the means $\pm$ SD of three independent experiments performed in duplicate. * $p < .05$.

Treatment of the NP-2 SCM with chondroitinase ABC but not heparinase removed the inhibitory effect (Fig. 5), suggesting that this inhibition might be mediated by CS molecules.

Discussion

In this study, we investigated the stability of HIV-1 incubated with different glioma cells since the CNS is an important organ, which is frequently infected with HIV-1 in the early stages of HIV-1 infection, resulting in many neurological abnormalities in AIDS patients.⁴⁴ We speculated that the brain cells may have additional features favoring HIV-1 infection in the CNS. For example, dendritic cells express a C-type lectin, DC-SIGN, that retains viral infectivity and enhances HIV-1 infection in *trans* to target cells.²¹

Previously, NP-2 and U87MG cell lines after transduction to express CD4 along with CXCR4 (NP-2/CD4/CXCR4 and U87/CD4/CXCR4 cells) or CCR5 (NP-2/CD4/CCR5 and U87/CD4/CCR5 cells) were shown to be highly susceptible to X4- and R5-HIV-1, respectively, although the parental NP-2 and U87MG cells were completely resistant to HIV-1 infec-

tion.^{20,45,46} Thus, it appears possible that NP-2 and U87MG cell lines may have some properties that contribute to retain HIV-1 infectivity and/or promote HIV-1 infection in the CNS.

Surprisingly, herein we found that HIV-1 titers decreased more rapidly after incubation with NP-2 or U87MG cells compared to HIV-1 incubated with A172, T98G, HeLa cells, or in cell-free culture medium alone (Fig. 1a). We demonstrated that HIV-1 binding to NP-2 or U87MG cells was similar, suggesting that the titer loss of HIV-1 upon incubation with NP-2 or U87MG cells was not due to the more attachment of HIV-1 to these glial cells (Fig. 2a). Moreover, we showed that NP-2 and U87MG cells secreted macromolecules that were present in the SCM of these cells and had the ability to suppress both R5- and X4-HIV-1 infection through inhibiting HIV-1 binding to target cells (Figs. 3a and 4b).

Although NP-2 and U87MG cells secrete molecules that are inhibitory for HIV-1 infection, NP-2/CD4/coreceptor or U87/CD4/coreceptor cells are highly susceptible to HIV-1 infection and have been widely used to test the coreceptor tropism of HIV-1.^{45,47} According to our experience, NP-2/CD4/coreceptor cells are even more susceptible to HIV-1 than

FIG. 5. Enzyme treatment of NP-2 SCM. NP-2 SCM and culture medium were treated with different concentrations of the chondroitinase ABC or heparinase (EC-4.2.2.7) enzymes at 37°C for 30 min, followed by enzyme inactivation by heating at 65°C for 10 min. The SCM were cooled on ice, mixed with HIV-1 Ba-L, and used to infect MAGIC-5A cells. The numbers of infected cells are shown. Treatment of the SCM with chondroitinase ABC but not heparinase abolished the inhibitory effect. The data are the means $\pm$ SD of three independent experiments performed in duplicate.

U87MG/CD4/coreceptor, HeLa/CD4/coreceptor, or U251/CD4/coreceptor cells (data not shown). Although culture supernatants from these HIV-1-susceptible NP-2 or U87MG cells infected with HIV-1 should contain the inhibitory molecules, the infectious titers of these culture supernatants could be as high as 1×10^5 infectious units per milliliter.

In the present study, the HIV-1 titers decreased only about 50% even when one volume of HIV-1 was incubated with nine volumes of SCM from NP-2 or U87MG cells (Table 1). Thus, the inhibitory activity of SCM seems not strong enough to restrict HIV-1 infection in these susceptible cells completely.

We have reported earlier that chondroitin sulfate type E (CS-E) is highly sulfated and is the most inhibitory among the CS molecules to both HTLV-1 and HIV-1.⁴² Although a concentration of CS-E as low as 50 μ g/ml is inhibitory to HTLV-1 infection, it cannot inhibit HTLV-1 infection completely, even at a very high concentration (90% inhibition at 400 μ g/ml).⁴² Unlike HTLV-1, HIV-1 infection is inhibited only 50% in the presence of 400 μ g/ml CS-E, suggesting that CS-E has a weak anti-HIV-1 activity.⁴² Similar to a high concentration of CS-E (400 μ g/ml), NP-2 SCM at a concentration as high as 90% volumes inhibited 50% of HIV-1 infection (Table 1). The escaped HIV-1 will easily spread among NP-2/CD4/coreceptor cells, although these cells may secrete inhibitory molecules.

Additive inhibitory effects were observed when HTLV-1 was treated with an HTLV-1 entry-inhibiting short peptide together with CS-E.⁴² Similarly, additive inhibitory effects on HIV-1 infection are expected when HIV-1 is treated with an HIV-1 entry inhibitor together with NP-2 SCM.

sGAG are frequently reported^{41,43} among the secretory molecules that can inhibit HIV-1 binding to target cells. Several cell lines, such as human B-cell lines,³⁸ breast cell lines (MDA-MB-231 and HBL-100),³⁹ and the NIH 3T3 cell line,⁴⁰ have been shown to secrete proteoglycans. Previously, Le Doux *et al.*⁴⁰ reported that proteoglycans secreted by NIH 3T3 cells inhibited retrovirus infection. Most of the inhibitory activity was attributed to factors >100 kDa and was sensitive to chondroitinase ABC digestion, which was consistent with our findings. Moreover, Newburg *et al.*⁴³ showed that human milk inhibited the binding of the HIV envelope glycoprotein gp120 to the cellular CD4 receptor, thereby suppressing HIV-1 infectivity. This inhibitory effect of human milk was also

lost following digestion with chondroitinase ABC but not heparinase I or heparinase III (heparitinase). These results are quite similar to our findings. We showed that the inhibitory factor secreted by NP-2 cells was eliminated by treatment with chondroitinase ABC but not heparinase (Fig. 5). Thus, we concluded that the inhibitory factor(s) secreted by NP-2 and U87MG cells was chiefly mediated by CS or CS-like moiety that may have inhibited gp120 binding to target cells.

In conclusion, our present study has clarified a single point: some but not all glioma cells secrete CS-like molecules that can inhibit HIV-1 infection to target cells. Although we used malignant cells here, both malignant and normal cells are often reported to secrete similar proteoglycans.³⁹ Thus, this study is helpful to understand the complex *in vivo* environment of the CNS for HIV-1 infection. Previous studies have reported that HIV-1 infection, production, and spread *in vivo* are much lower than those observed *in vitro*.⁸ Our present study suggests that HIV-1-resistant cells surrounding HIV-1-susceptible cells may secrete inhibitory molecules that play an important role in lowering HIV-1 infection *in vivo*. The data reported herein also suggest that the CS-like molecules are predominant naturally occurring glycoconjugates that inhibit HIV-1 binding to host cells.

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Author Disclosure Statement

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