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***In vitro* Glutaminase Regulation and Mechanisms of Glutamate Generation in HIV-1 Infected Macrophage**

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Abstract

Mononuclear phagocyte (MP, macrophages and microglia) dysfunction plays a significant role in the pathogenesis of HIV-1-associated dementia (HAD) through the production and release of soluble neurotoxic factors including glutamate. Glutamate production is greatly increased following HIV-1 infection of cultured MP, a process dependent upon the glutamate-generating enzyme glutaminase. Glutaminase inhibition was previously found to significantly decrease macrophage-mediated neurotoxicity. Potential mechanisms of glutaminase-mediated excitotoxicity including enzyme upregulation, increased enzyme activity and glutaminase localization were investigated in this report. RNA and protein analysis of HIV-infected human primary macrophage revealed upregulation of the glutaminase isoform GAC, yet identified no changes in the KGA isoform over the course of infection. Glutaminase is a mitochondrial protein, but was found to be released into the cytosol and extracellular space following infection. This released enzyme is capable of rapidly converting the abundant extracellular amino acid glutamine into excitotoxic levels of glutamate in an energetically favorable process. These findings support glutaminase as a potential component of the HAD pathogenic process and identify a possible therapeutic avenue for the treatment of neuroinflammatory states such as HAD.

Keywords

HIV-1-associated dementia; macrophages; glutamate; glutaminase

Introduction

HIV-1 Associated Dementia (HAD) results from prolonged inflammation in the CNS and is a significant consequence of HIV infection resulting in a chronic, progressive dementia. Mononuclear phagocytes (MP) are critical to HAD pathogenesis and have been hypothesized to induce neuronal injury through the production and release of various soluble

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neurotoxic factors including glutamate (Belmadani *et al.* 2001, Zink *et al.* 1999, Giulian *et al.* 1993, Pulliam *et al.* 1994, Jiang *et al.* 2001). Glutamate mediates numerous vital physiological functions through activation of multiple receptors (Cutler & Dudzinski 1974, Fonnum 1984, Orrego & Villanueva 1993); however, high concentrations of extracellular glutamate induce neuronal damage (Olney 1971, McCall *et al.* 1979, Choi 1988, Newcomb *et al.* 1997). HIV-1-infected patients have significantly higher concentrations of glutamate in their plasma and cerebrospinal fluid as compared to uninfected controls (Ollenschlager *et al.* 1988, Droge *et al.* 1987, Ferrarese *et al.* 2001) and HIV-1 infected macrophages are an important cellular source of extracellular glutamate (Jiang *et al.* 2001).

Phosphate-activated glutaminase (PAG, EC 3.5.1.2) is the primary enzyme for the production of glutamate (Wurdig & Kugler 1991, Nicklas *et al.* 1987, Ward *et al.* 1983, Curthoys & Watford 1995) and is also the predominant glutamine-utilizing enzyme of the brain (Kvamme *et al.* 1982, Holcomb *et al.* 2000). Glutaminase is generally localized to the inner membrane of the mitochondria and catalyzes the deamination of glutamine to glutamate, a hydrolysis resulting in stoichiometric amounts of glutamate and ammonia (Shapiro *et al.* 1985, Shapiro *et al.* 1991, Laake *et al.* 1999). We previously identified generation of the glutamate by HIV-1 infected human monocyte-derived macrophage (MDM) (Zhao *et al.* 2004). The increase in glutamate is neurotoxic and represents a major contribution to macrophage-mediated neurotoxicity (Tian *et al.* 2008a). Excess glutamate production is dependent upon productive infection as well as the presence of glutamine (Zhao *et al.* 2004). We recently demonstrated glutaminase activity is required for glutamate production, and that glutamine removal, glutaminase specific siRNA, and small-molecule glutaminase inhibitors all effectively prevent excess glutamate production (Erdmann *et al.* 2007). While glutaminase function is required for glutamate production, the mechanism responsible for this excess generation is unclear. An increase in glutaminase amount, activity or release of enzyme mediated by the infective process of HIV-1 may facilitate uncontrolled generation of glutamate in the CNS.

Kidney-type glutaminase (KGA), found on chromosome two in humans (Mock *et al.* 1989) has various isoforms generated through tissue specific alternative splicing. KGA is abundant not only in the kidney, but also the brain, intestine, lymphocytes and various tumors (Curthoys & Watford 1995). Elgadi *et al.* first described GAC, a KGA isoform known to be present in the brain (Elgadi *et al.* 1999). GAC mRNA is produced by alternative splicing of a single exon within the KGA gene (Porter *et al.* 2002), the resulting protein shares much of the functional KGA regions, but GAC contains a unique 3' tail. The first 16 N-terminal amino acids of KGA and GAC encode a mitochondrial targeting sequence (Porter *et al.* 1995) and glutaminase is found almost exclusively in the mitochondria. Here, we characterize the expression of the glutaminase isoforms KGA and GAC in monocyte-derived macrophages (MDM) during HIV infection. Furthermore, we identify the release of glutaminase as a possible mechanism of glutaminase-mediated production of excitotoxic glutamate during HIV-infection.

Materials and Methods

Isolation and culture of monocyte-derived macrophages (MDM)

Human monocytes were recovered from peripheral blood mononuclear cells of HIV-1, -2 and hepatitis B seronegative donors after leukaphoresis, and then purified by counter current centrifugal elutriation as previously described (Gendelman *et al.* 1988). After seven days of culture in the presence of M-CSF (macrophage colony stimulating factor; a generous gift from Genetics Institute, Inc., Cambridge, MA) monocytes were considered MDM. All tissue reagents were screened and found negative for endotoxin (< 10 pg/mL; Associates of Cape Cod, Inc., Woods Hole, MA) and mycoplasma contamination (Gen-probe II; Gen-probe

Inc., San Diego, CA). Seven days after plating, MDM were infected with HIV-1 strains ADA, JR-FL, or 89.6 at a multiplicity of infection (MOI) of 0.1 virus/target cell. Viral stocks were screened for mycoplasma and endotoxin using hybridization and limulus amebocyte lysate assays, respectively.

Affymetrix Array

After isolation using TRIzol, mRNA from triplicate HIV-1_{ADA}-infected and uninfected MDM was used to generate tagged cDNA, which was hybridized to an Affymetrix GeneChip HG-U133A Array. The HG-U133A Array includes representation of the RefSeq database sequences and probe sets related to sequences previously represented on the Human Genome U95Av2 Array. It contains 22,283 human gene probe sets representing about 14,500 genes. The cell intensities from the 11-20 probe pairs for each probe set (gene) were analyzed by means of MAS 5.0 to calculate a single numerical gene expression value representing gene expression abundance.

RNA extraction and TaqMan real-time RT-PCR

Total RNA was isolated with TRIzol Reagent (Invitrogen Corp, Carlsbad, CA, USA) and RNeasy Mini Kit according to the manufactures' protocol (Qiagen Inc., Valencia, CA, USA). Assays-on-Demand primers for GAC (ID# 528445), KGA (ID# 489954) and GAPDH (ID#, 4310884E) were purchased from Applied Biosystems Inc (Foster City, CA, USA). Real-time reverse-transcription polymerase chain reaction (real-time RT-PCR) was carried out using the one-step quantitative TaqMan Real-time RT-PCR system (Applied Biosystems Inc., Foster City, CA, USA). Relative KGA and GAC mRNA levels were determined and standardized with a GAPDH internal control using comparative $\Delta\Delta CT$ method. All primers used in the study were tested for amplification efficiencies and the results were similar.

Western blot analysis of Glutaminase

Proteins from lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoretic transfer to polyvinylidene difluoride (PVDF) membranes (Millipore and Bio-Rad), proteins were treated with purified primary antibodies (VDAC, KGA, GAC and β -actin) overnight at 4°C followed by a horseradish peroxidase-linked secondary anti-rabbit antibody (1:5000 dilution; Cell Signaling Technologies). Antigen-antibody complexes were visualized by enhanced chemiluminescence western blotting on Hyperfilm ECL (Amersham). For data quantification the films were scanned with a CanonScan 9950F scanner; the acquired images were then analyzed on a Macintosh computer using the public domain NIH image program (developed at the U.S. National Institutes of Health and available on the internet at <http://rsb.info.nih.gov/nih-image/>).

siRNA knockdown of Glutaminase

siRNA knockdown in MDM was performed as previously described (Peng *et al.* 2006). Briefly, pre-designed siRNA duplexes targeted against glutaminase mRNA were synthesized by Dharmacon (Lafayette, CO). MDM were infected with HIV-1_{ADA} at a multiplicity of infection (MOI) of 0.1 virus/target cell. Two days post-infection cells were transfected with 100 nM siRNA duplex for 24 h in the presence of siIMPORTER (Upstate Cell Signaling Solutions, Charlottesville, VA) according to the manufacturer's instructions. A non-specific control siRNA (Dharmacon, Lafayette, CO) was also transfected at the same concentration as a control.

Analyses of glutamate and glutamine by RP-HPLC

HPLC analysis was performed using an HP Series II 1090 liquid chromatograph and HP1046A fluorescence detector (Hewlett Packard) as previously described (Zhao et al. 2004).

MTT reduction assay

Cell viability was assessed by MTT reduction as described previously (Jiang et al. 2001, Zhao et al. 2004).

Rat Brain Mitochondrial Isolation

Rats (Sprague Dawley) were sacrificed by decapitation. Brain mitochondria isolation was conducted according to previously described methods (Tian *et al.* 2008b). In brief, rat brain tissue was homogenized and then centrifuged at 2,000 g for 10 min in MSETB buffer (210 mM mannitol, 70 mM sucrose, 0.5 mM ethylenediamine tetra-acetate, 10 mM Tris-HCl and 0.2% bovine serum albumin, pH7.4). The suspension was then centrifuged at 16,000g for 10 min before being washed in SET buffer (280 mM sucrose, 0.5 mM EDTA and 10 mM Tris-HCl, pH 7.4). Mitochondrial samples were then isolated after centrifugation at 16,000g for 8 min. Equal mitochondrial fractions were treated with different concentrations of hydrogen peroxide (H₂O₂) at 25 °C in PT-1 buffer containing 250 mM sucrose, 2 mM HEPES, pH 7.4, 0.5 mM KH₂PO₄, 2 μM rotenone and 4.2 mM potassium succinate, for 60 min, in addition, 10 μM cyclosporine A (CsA) was pre-incubated with mitochondria for 10 min, then H₂O₂ was added and incubated for the same time. The samples were then centrifuged at 12,000×g for 15 min at 4 °C. The supernatants were analyzed by Western blotting for glutaminase, and VDAC was used as a loading control.

Cell Fractionation

Cells were fractionated by differential centrifugation as described previously (Tian et al. 2008b). Briefly, cells were harvested through trypsin digestion, and then centrifuged and resuspended in three volumes of hypotonic buffer (210 mM sucrose, 70 mM mannitol, 10 mM HEPES (pH 7.4), 1 mM EDTA) containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). After gentle homogenization with a Dounce homogenizer, cell lysates were centrifuged at 1 000 g for 5 min to remove unbroken cells and nuclei and the cytosolic fractions were obtained by further centrifugation at 10 000 g for 30 min.

Statistical analysis

Data were analyzed as means ± standard deviation (SD). The data were evaluated statistically by the analysis of variance (ANOVA), followed by the student's *t*-test for paired observations. Significance was determined as *p*<0.05, *p*<0.01 and *p*<0.001.

Results

Glutaminase isoforms and their regulation

Elutriated human monocytes were differentiated for seven days into MDM then infected with HIV-1_{ADA}. After 5 days of infection, RNA collected from control and HIV-infected MDM was applied to an affymetrix array to evaluate global RNA regulation. This preliminary microarray analysis of infected MDM revealed regulation of a specific glutaminase isoform. Although KGA had no apparent RNA regulation, the GAC isoform was upregulated (Fig. 1A). Our previous studies identified glutaminase as essential to the generation of excess glutamate following HIV-infection of MDM (Erdmann et al. 2007). Glutaminase has two primary isoforms, liver-type glutaminase (LGA) and kidney-type glutaminase (KGA). LGA is present in the CNS but at relatively low expression levels as

compared to KGA (Baglietto-Vargas *et al.* 2004). KGA is located on chromosome 2 and has 19 exons. The primary KGA isoform includes exons 1-14 and 16-19, with exon 15 spliced out. The GAC isoform originates from the same locus, but includes exons 1-15, and thus has a unique C-terminus (Fig. 1B). The functional role of each isoform is unclear, however the arrangement of the locus allows for specific regulation of KGA and GAC.

Expression of Glutaminase Isoforms During HIV-Infection

Real-time RT-PCR was used to quantify the expression of glutaminase isoforms KGA and GAC in infected MDM over the course of infection. Probes were designed specific to the C-terminus of KGA and GAC respectively. As the infection progressed from day 1 through day 9 (Fig. 2A), expression of the KGA isoform did not significantly change as demonstrated by the representative donor (Fig. 2B). The GAC isoform lacked significant regulation at days 1 and 3 post-infection, but was significantly upregulated on days 5, 7 and 9 as compared to control (Fig. 2C). The GAC upregulation peaked at day 7, and was expressed 7.9 fold higher in HIV-infected MDM as compared to control. We also used macrophage-tropic HIV-1 strains HIV-1_{JR-FL} and dual-tropic HIV-1_{89.6} to infect human MDM as a comparison to HIV-1_{ADA}. Seven days after infection, culture supernatants were monitored for HIV-1 viral infectivity using the reverse transcriptase activity assay (Fig. 2D). All tested viral strains significantly increased GAC expression levels, although HIV_{ADA} induced the highest increase of GAC (Fig. 2E). To further demonstrate the relationship of GAC expression and HIV infection, two representative donors were treated with Zidovudine (AZT, HIV-1 reverse transcriptase inhibitor) and RNA was collected 5 days post-infection (Fig. 2F). The significant upregulation of GAC was blocked by AZT treatment, indicating a dependence on productive infection.

Glutaminase is responsible for significantly increased production of glutamate and RNA analysis indicated regulation of the GAC isoform during HIV infection. We next measured protein levels through Western blotting using antibodies specific to the C-terminals of KGA and GAC. In the representative donor presented below, KGA shows no significant change in protein levels between control and HIV-infected MDM at any time point (Fig. 3A, C). The findings for KGA are consistent with the absence of RNA regulation. Using a GAC specific antibody, upregulation was observed at days 5, 7 and 9, peaking with a 2-fold increase at 5 days post-infection (Fig. 3A, B). The phenomenon was further demonstrated following AZT treatment where the GAC isoform is enhanced following HIV-infection, but is prevented by anti-retroviral treatment (Fig. 3D, E).

siRNA knockdown of GAC Isoform

We applied siRNA to specifically knockdown the GAC isoform of glutaminase. MDM were infected for two days before being transfected with either non-specific siRNA or siRNA targeting the GAC specific C-terminus. Three days post-siRNA transfection, MCM and protein were collected from control and infected macrophages. Western blot analysis demonstrated a decrease in glutaminase protein by GAC siRNA (Fig. 4A, B), whereas the levels of KGA were not affected by GAC siRNA (Fig. 4A, C). As measured by RP-HPLC, glutamate levels in macrophage-conditioned media (MCM) were found to be significantly decreased by GAC siRNA in infected macrophage cultures as compared to non-specific siRNA-transfected HIV culture (p value <0.01, Fig. 4D).

Glutamate production by HIV-1-infected MDM and the effect of cytotoxicity

We evaluated the production of glutamate by MDM following treatment with the cytotoxic agent staurosporine (STS) with or without HIV-1 infection. Glutamate concentrations in MCM were measured by RP-HPLC 7 days post-infection (Fig. 5A). MCM collected from infected cell cultures contained significantly higher amounts of glutamate as compared to

MCM from uninfected cells. Treatment with STS induced elevated glutamate levels, however a relatively high dose was required (1 mM) and the glutamate production was not equal to that produced by HIV-infection alone. STS treatment in addition to HIV-infection led to a synergistic enhancement of glutamate production. Cell viability was assessed by MTT assay following stimulation (Fig. 5B). These findings indicate glutamate production is enhanced by cell death, however, cell death alone does not lead to the levels of glutamate observed during HIV infection.

Mitochondrial Release of Glutaminase *ex vivo*

Mitochondria are a focal point of cell death processes and are known to be affected during HIV infection. The full mRNA transcripts of both KGA and GAC glutaminase include a mitochondrial localizing sequence, and nearly all glutaminase is localized in the mitochondria. Destabilization of the mitochondrial membrane is known to lead to the release of small proteins such as cytochrome C through the mitochondrial transitional pore complex (Hansson *et al.* 2003). *Ex vivo*, we tested whether glutaminase was released from intact mitochondria following oxidative stress. Intact rat brain mitochondria were isolated and then stimulated with increasing levels of hydrogen peroxide. Using a glutaminase specific antibody, we identified glutaminase in the supernatants of stimulated mitochondria (Fig. 6A). The amount of observed glutaminase increased as the amount of hydrogen peroxide used for stimulation was increased (Fig. 6B). The mitochondrial stabilizing agent CsA treatment reduced the amount of glutaminase released to the supernatants. The presence of glutaminase in the supernatants of the rat brain mitochondria demonstrated the potential for glutaminase to be released from mitochondria following stress or damage.

We next isolated mitochondrial and cytosolic fractions from control and infected MDM and determined the amount of glutaminase present with Western blotting. Glutaminase levels in the mitochondrial fractions indicated an increase in the HIV group, consistent with whole-cell measurements (Fig. 7A, B). The cytosolic fraction from uninfected MDM had detectable levels of glutaminase present despite the presence of some of the mitochondrial marker VDAC. However, the cytosol fraction from infected MDM had significantly enhanced levels of glutaminase (Fig. 7A, C). These findings indicate the process of HIV-infection causes a release of glutaminase from mitochondria into the cytosolic compartment of infected cells.

HIV-infection of macrophages leads to formation of giant multi-nucleated cells in addition to cell damage and death at late stages of infection. Thus, release of glutaminase from the mitochondria to the cytosol may be a direct precursor to release of functional glutaminase enzyme to the extracellular space. The principle of glutaminase release was tested *in vitro* through cultures of control and HIV-infected MDM. Equal amounts of control and HIV conditioned medium were collected from cultures 7 days post-infection and precipitated with trichloroacetic acid (TCA). Protein pellets were resuspended and glutaminase was quantified via Western blotting (Fig. 7D). Although there was minimal glutaminase present in control samples, a significant amount of GAC was present in HIV conditioned media (Fig. 7D). These findings indicate glutaminase is upregulated and released to the extracellular space in MDM following HIV-infection.

Discussion

We have previously reported an HIV-mediated increase in glutamate production by MDM (Zhao *et al.* 2004, Erdmann *et al.* 2007). In this report, RNA regulation of the predominant glutaminase isoform KGA was found to be unchanged, but the glutaminase isoform GAC was significantly increased at later stages of HIV-1 infection (Fig. 1 and 2). These findings were supported by Western blotting of glutaminase protein where the GAC isoform was

found to be modestly increased (Fig. 3). siRNA targeting of the GAC isoform significantly decreased glutamate production, indicating the relevance of the GAC isoform to pathogenesis (Fig. 4). Glutamate production by MDM increased as HIV infection progressed, further, the addition of cytotoxic agents enhanced glutamate production, particularly in HIV-infected cell populations (Fig. 5). This finding supported the hypothesis that cellular damage may enhance glutamate generation. Using subcellular analysis, we identified release of glutaminase from mitochondria *ex vivo* from intact rat brain mitochondria validating the glutaminase release model (Fig. 6). Glutaminase release was then observed *in vitro* in the cytosolic fractions of infected MDM (Fig. 7A), as well as in the conditioned medium of infected macrophage cultures (Fig. 7D). Cumulatively, we characterized glutaminase regulation in HIV-1-infected MDM and identified release of glutaminase from mitochondria *ex vivo* and *in vitro* identifying a potential mechanism of excess glutamate production.

Excitotoxicity is a fundamental component of various neurodegenerative disorders. In HAD, enhanced susceptibility of neuronal populations, alterations in astrocyte function, and increased presence of excitotoxins combine to generate an excitotoxic environment in the CNS. Although a variety of factors clearly contribute to HAD pathogenesis, glutamate appears to be a critical component of HAD excitotoxicity. In HAD, significant numbers of mononuclear phagocytes migrate into the CNS where they are activated and/or productively infected. Glutamate is secreted in large quantities by macrophage (Piani *et al.* 1991, Zhao *et al.* 2004, Jiang *et al.* 2001), and glutaminase is expressed at significant levels by MDM (Zhao *et al.* 2004). Glutaminase converts glutamine to glutamate in an energetically favorable process. Glutamine is widely available in the CNS, typically in the millimolar range in cerebrospinal fluid. Increased glutamate is dependent upon the presence of glutamine and glutaminase activity. HIV-1 infection of human macrophages leads to a drastic and potentially pathogenic increase in glutamate (Tian *et al.* 2008a).

Viral infection leads to formation of multinucleated giant cells, as well as mitochondrial stress. This cellular stress has the potential to disrupt membrane stability leading to release of mitochondrial glutaminase. Our group demonstrated a glutamine-dependent upregulation of glutamate production by HIV-1 infected macrophage cultures. This glutamate increase related to cell viability, and was nearly eliminated in the presence of antiviral treatment (Zhao *et al.* 2004). We hypothesized HIV-1 infection may lead to increased enzyme activity or release of enzyme into a glutamine rich substrate with little product feedback, allowing excess glutamate generation from macrophage populations. In HAD, immune cell recruitment, activation and infection causing cell stress and death may then lead to poor regulation of glutaminase, producing an excitotoxic environment.

After observing increased glutamate production by HIV-infected MDM, we identified the enzyme glutaminase was required for this phenomenon (Erdmann 2007). Glutaminase protein upregulation was a straightforward hypothesis and our initial primary focus, but preliminary studies failed to identify any mRNA or protein regulation of KGA. Previous studies have identified inflammatory factors, notably TNF- α , have the ability to increase glutaminase levels in microglia (Yawata *et al.* 2008). Further, the glutaminase locus is located immediately next to Stat1 on chromosome 2, a predominant pathway of HIV-induced inflammation supporting the potential for glutaminase upregulation during acute inflammation. We were unable to observe any mRNA changes of KGA or GAC following inflammatory factor stimulation, including TNF- α and LPS (Data not shown). Further, we have also tried to treat human MDM with gp120 (.1-10 nM) and have not observed a significant increase in glutamate production. We were however able to consistently observe GAC upregulation at later stages of HIV-1 infection in human MDM. Which component of the HIV-1 infectious process in MDM is responsible for the regulation of glutaminase and

the influence of the *in vitro* environment is not known. The overall significance of the glutaminase isoforms is still unclear, but GAC regulation has recently been observed in a variety of tumors (Szeliga *et al.* 2008), indicating the GAC isoform is possibly regulated in an active fashion whereas KGA is constitutively expressed.

Despite the significant regulation of GAC mRNA, up to 7 fold (Fig. 2), the change in protein levels is relatively modest (Fig. 3). This 2-fold upregulation of protein levels is not likely solely responsible for the vast increase in glutamate levels observed in culture, particularly in situations of enhanced cell damage and death (Fig. 5). We tested the glutamate generating capacity of glutaminase from uninfected MDM as compared to glutaminase from infected MDM and found no significant difference (Data not shown). We then investigated the compartmentalization of glutaminase following infection. Glutaminase is almost exclusively located in the mitochondria, but we found intact rat brain mitochondria released glutaminase in response to oxidative stress (Fig. 6).

After demonstrating the ability of glutaminase to be released from stressed mitochondria *ex vivo*, we tested whether glutaminase was released in primary human MDM. Using the cytosolic fraction of HIV-1-infected MDM as an intermediate compartment for glutaminase release, we found a significant increase in glutaminase levels (Fig. 7A). This increase in glutaminase levels was not present in control samples, indicating HIV-1 infection was required for glutaminase release. After precipitating the protein in conditioned medium, glutaminase was also found in the extracellular space of infected MDM (Fig. 7D). In the human MDM studies, a GAC specific antibody was used to identify glutaminase release into the cytosol and extracellular space. The KGA-specific antibody was unable to detect glutaminase in conditioned-medium of HIV-1-infected cells, but did identify modest KGA release to the cytosolic compartment. These findings indicate the GAC isoform may be upregulated and may be preferentially released, however technical shortcomings in identifying KGA release cannot be ruled out at this time.

Following the significant disruption of cell homeostasis induced by productive HIV-infection, we observed glutaminase in the conditioned medium of HIV-infected cultures of MDM. We were however, unable to observe glutaminase in the conditioned-medium from control cultures. Upon release of glutaminase from mitochondria and into the extracellular space, the enzyme is exposed to high levels of substrate, glutamine. The specific effects of HIV-1 infection on MDM are not completely known. Macrophages are readily infected during HIV-1 infection and provide a latent viral reservoir. These cells are typically resistant to cell death unlike infected T-cell populations. However, a variety of factors have been identified that alter survival processes or disrupt mitochondrial function. Cellular stress, particularly those altering mitochondrial homeostasis such as reactive oxygen species may destabilize the mitochondrial membrane facilitating glutaminase release. We have shown the GAC isoform of glutaminase is upregulated in HIV-1-infection of MDM and is released from the mitochondria *in vitro*. The relative contribution of glutaminase to neuropathology *in vivo* is still unclear, as is the importance of the GAC isoform specifically.

Because glutaminase may be contributing to neuronal damage through glutamate toxicity, the ability to block its function may provide a therapeutic avenue in a variety of diseases where excitotoxicity is prominent. In HAD, multiple pathways combine to sensitize neuronal populations and generate excitotoxic insults (Erdmann N 2006). Although NDMA mediated Ca^{2+} influx is a fundamental component of neuronal excitotoxic damage, preventing NMDA receptor stimulation with agents such as MK-801 cause serious side effects and are not a viable therapeutic approach; however, partial blockade of NDMA receptors with the drug memantine has shown therapeutic benefit with limited complications (Chen & Lipton 2006, Anderson *et al.* 2004). Targeting all glutaminase is unacceptable due

to its vital roles in not only generation of glutamate as a neurotransmitter, but also its contribution to cellular metabolism as was evidenced by recent knockout studies (Masson *et al.* 2006). Although glutaminase is critical to normal brain function, the phenomenon presented here indicates HIV-1 mediated dysfunction of glutaminase facilitating uncontrolled glutamate generation. Through the determination of the mechanisms involved in this process, and development of glutaminase inhibitors such as those tested in our previous work (Erdmann 2007), glutaminase may emerge as a viable therapeutic target of neurodegenerative disorders.

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A.

Microarray analysis of glutaminase isoforms expression on two donors

B.

KGA

GAC

5' Untranslated Region

Shared cDNA

Unique 3' cDNA

3' Untranslated Region

Figure 1. Glutaminase isoform regulation identified by microarray analysis

Panel A, Human MDM were infected with HIV-1_{ADA} for 5 days before total RNA was collected. mRNA from duplicate HIV-1_{ADA}-infected and uninfected MDM was used to generate tagged cDNA, which was hybridized to an Affymetrix GeneChip HG-U133A Array. Hybridization intensities for each probe set (gene) were analyzed by means of MAS 5.0 to calculate a single numerical gene expression. Panel B, representation of KGA and GAC mRNA transcripts. Both genes are encoded at the same locus, mRNA is processed yielding two distinct gene products. KGA protein is encoded by exons 1-14 and 16-19. GAC is encoded by exons 1-15 and consequently has a unique C-terminus. The functional region of glutaminase enzyme is shared by both gene products.

Figure 2. Real-time RT-PCR of glutaminase during course of HIV infection

MDM were infected with HIV-1_{ADA} for 1, 3, 5, 7 and 9 days before total RNA was collected. A, Supernatants were tested for reverse transcriptase activity. B-C, Real-time probes specific to KGA glutaminase (B) or GAC glutaminase (C) were applied to determine expression levels as compared to the internal control GAPDH. D-E, MDM were infected with HIV-1_{ADA}, HIV-1_{JR-FL}, or HIV-1_{89.6}, culture supernatants and RNA were collected 7 days after infection and reverse transcriptase activity (D) and GAC expression (E) were determined. Results are expressed as average $\pm$ SD of triplicate samples and are representative of 3 independent experiments with MDM from at least 3 different donors. * denotes $p < 0.01$ in comparison to control. In panel F, cells were infected with HIV-1_{ADA} with or without AZT (5 μ m) treatment for 7 days. GAC expression was determined by real-time RT-PCR. * denotes $p < 0.01$ in comparison to control, # denotes $p < 0.05$ as compared to HIV infection alone.

Figure 3. Western blotting of glutaminase during course of HIV-1 infection

Human MDM were grown in culture and then infected with HIV-1_{ADA} for 1, 3, 5, 7, or 9 days. A, Whole cell lysates were collected and analyzed by Western blot for glutaminase (KGA and GAC). β -actin was used as a loading control. B-C, Levels of GAC (B) and KGA (C) were normalized as a ratio to β -actin after densimetrical quantification and shown as percentage of control (1 dpi). Open bars represent control MDM and solid bars represent HIV-1-infected MDM. D-E, MDM were infected with HIV-1_{ADA} for 7 days with or without AZT treatment (5 μ M). Whole-cell lysates were collected and analyzed by Western blotting for GAC (D). GAC Bands were quantified and expressed as percentage of control (E). Quantification results were shown as average $\pm$ SEM in experiments performed with 4 different donors. *, $p < 0.05$ compared with day-matched control. # indicates $p < 0.05$ when compared to HIV group.

Figure 4. siRNA treatment targeting GAC in infected human macrophage

Human MDM were grown in culture and infected with HIV-1_{ADA}. Infected MDM were then transfected with siRNA targeting glutaminase or non-specific control. A.) Whole cell lysates were collected and analyzed by Western blotting for glutaminase (KGA and GAC). β-actin was used as a loading control. B-C, Levels of GAC (B) and KGA (C) were normalized as a ratio to β-actin after densimetrical quantification and shown as percentage of control. Quantification results were shown as average ± SEM in experiments performed with 4 different donors. * denotes $p < 0.05$ compared with control MDM. ## indicates $p < 0.01$ when compared to HIV group. D.) Supernatants were analyzed by RP-HPLC for glutamate concentration. Results are expressed as average ± SEM of 2 independent experiments with MDM from 2 different donors. ** denotes $p < 0.01$ compared with control or non-specific siRNA control. ## indicates $p < 0.01$ when compared to HIV or non-specific siRNA-transfected HIV group.

Figure 5. HIV-1-mediated glutamate production enhanced by cytotoxicity

Human MDM were infected with HIV-1_{ADA} for 7 days and then incubated in serum-free neurobasal medium with 5 mM glutamine. Control and HIV-infected MDM were treated with staurosporine (1, 5, or 10 mM). The concentration of glutamate in cell-free supernatants was determined by RP-HPLC (A). All data are expressed as the absolute concentration of glutamate (μM). * denotes $p < 0.01$ in comparison to control. # denotes $p < 0.01$ as compared to HIV infection alone. Cell viability was assessed by MTT assay (B). Results are expressed as average $\pm$ SD of triplicate samples and are representative of 3 different donors. * denotes $p < 0.01$ in comparison to control, ** denotes $p < 0.001$.

Figure 6. Glutaminase release from rat brain mitochondria

Rat brain mitochondria were isolated and stimulated ex vivo with H₂O₂ (0.1, 0.5, or 1 mM) with or without cyclosporine A (5 μ M) treatment. A, Mitochondrial supernatants were collected and analyzed via Western blotting for glutaminase and the mitochondrial loading control VDAC. B, Levels of glutaminase were normalized as a ratio to VDAC after densimetrical quantification and shown as fold change to control. Results are expressed as average $\pm$ SEM of 2 independent experiments with rat brain mitochondria from 2 different donors. * denotes $p < 0.05$ compared with control. ## indicates $p < 0.01$ when compared to HIV group.

Figure 7. Glutaminase release from human MDM mitochondria *in vitro*

Human MDM were infected with HIV-1_{ADA} for seven days in culture. (A) Cells were collected and separated into mitochondrial and cytosolic fractions. Fractions were analyzed by Western blotting for GAC, VDAC and β -actin. VDAC and β -actin were used as loading controls for mitochondrial and cytosolic fractions, respectively. B-C, Levels of GAC in mitochondria (B) and cytosol (C) were normalized as a ratio to VDAC or β -actin after densitometrical quantification and shown as percentage of control. In panel D, Protein from equal volumes (30 ml) of conditioned-medium from cultures control or HIV-infected MDM were precipitated using TCA. GAC levels in the precipitated protein were determined by Western blotting. Results are representative of 3 different donors. Quantification results were shown as average $\pm$ SEM in experiments performed with MDM from 4 different

donors. *denotes $p < 0.05$ compared with control. ** indicates $p < 0.01$ compared with control.