Rapid Communication

Chloroquine mediated molecular tuning of astrocytes for enhanced permissiveness to HIV infection

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A R T I C L E   I N F O

Article history:
Received 8 May 2008
Returned to author for revision 2 June 2008
Accepted 29 July 2008
Available online 11 September 2008

Keywords:
HIV-astrocyte
Lyso-somotropic agents
HIV-reservoir

A B S T R A C T

We report in this study that minimum productive HIV infection in astrocytes (a predominant cell type in brain and persists for the entire life) occurs through endocytosis. The lysosomal agent chloroquine enhanced permissiveness of astrocytes to HIV infection possibly by circumventing degradation of endosome-entrapped viral particles. In particular, chloroquine may promote establishment of a stable long term viral reservoir in astrocytes and may eventually facilitate early onset of neurological complications.

Introduction

HIV infection of the brain may lead to cognitive and motor impairment and may eventually cause a dementing illness. The brain is also an important reservoir for HIV where it resides in macrophages/microglia and astrocytes. The life cycle of the HIV in these two cell types is different. In macrophages, HIV enters through a pH-independent pathway using the CD4-receptor and coreceptor either CCR5 or CXCR4, leading to fusion mediated viral entry (Fackler and Peterlin, 2000). In astrocytes, the mechanism of HIV entry is not completely understood; however, it is known to be independent of CD4-receptor (Tornatore et al., 1994) and is shown to be via endocytosis leading to minimum productive infection (Li et al., 2007). This mode of viral entry is also used for productive infection by other enveloped viruses (Chandran et al., 2005).

Chloroquine is known to have a profound influence on endosomes and causes reprogramming of endosomal pH (Maxfield, 1982; Wei et al., 2005). It is also widely used as an anti-malarial drug in regions of the world where HIV and malaria are co-existent. Chloroquine (10–25 μM) has been shown to confer a weak anti-HIV activity outside the range of physiological concentrations by disrupting the glycosylation of gp120 (Rayne et al., 2004; Naarding et al., 2007). None the less, some reports have demonstrated no beneficial effect of chloroquine and instead have shown increased HIV infection in vitro and a more severe form of disease outcome in brain with upregulation of viral replication of other neurotropic viruses (Kamya et al., 2001; Seth et al., 1999). Given their high endocytic activity for HIV particles (Clarke et al., 2006; Deiva et al., 2006), in the present study we investigated the impact of chloroquine on HIV infection in neuro-glial cells.

Results and discussion

Human fetal astrocytes (HFA) from aborted fetuses of 8–10 weeks age were obtained according to institutional guidelines and were cultured in 6–24 wells culture plates as described earlier (Chauhan et al., 2007). The HFA were infected with recombinant HIV (NLENY1) containing an YFP-gene inserted between the nef and env viral genes (Kutsch et al., 2002). The virus was prepared in 293-T cells upon transfection of either NLENY1 DNA alone or in combination with VSV-G expression plasmid followed by collection of supernatants after 72 h and treatment with 100 U of RNase free DNase for 20 min at RT (Chauhan et al., 2007). The toxicity dose response concentrations were from 0–500 μM (Fig. 1) and concentrations at or above 12.5 μM were found to be toxic on HFA in 24–48 h of treatments. The plasma concentrations of chloroquine is 0.1 μM while in lung, heart and kidneys is 200 times and in brain is 10–30 times of plasma levels with a half life of 7–10 days. Hence, chloroquine was used within the physiological range achievable in brain. Following treatment with chloroquine (0–5 μM) for 2 h either pre- or post-HIV infection, HFA cultures were monitored for infection. However, 5 μM concentration was found to be toxic in long term culture treatments as well as was outside the physiological limit. To keep the concentration within upper physiological range (brain), chloroquine was used at 2.5 μM without any toxicity in all the experiments either short term (2 h) or long term (4–21 days). In parallel as controls, HIV infection studies were performed on HFA using a specific lysosomotropic agent Bafilomycin-A. The infection was followed up for 3 weeks, a time...
period based on our previous HIV infection baseline studies on neuroglial cells (Chauhan et al., 2007; Chauhan et al., unpublished]. The HIV-infected astrocytes were tracked and counted by YFP-fluorescence every 5 days. The supernatants collected at various times were analyzed quantitatively for viral replication by p24 assay (ZeptoMetrix Corp., NY, USA). The HIV-infected cells were identified as astrocytes upon immunostaining using monoclonal antibody to GFAP (Sigma, USA), a marker for astrocytes. The HIV NLENY1-infected Jurkat cells were used as controls to compare the overall infection.

An extremely low level of productive infection (less than 0.025%) by HIV-NLENY1 virus was observed in HFA compared to Jurkat cells (Fig. 2A). Intriguingly, upon chloroquine treatment, a several fold (15–20 folds) increase in HIV infection in a dose dependent manner (data only shown for 2.5 μM concentration) was observed in comparison to untreated but infected astrocytes (Figs. 2A and B). In parallel with chloroquine and bafilomycin-A, minocycline was used as a control which does not interfere in endocytosis and endosomal pH. Minocycline has shown to confer anti-HIV activity. In our experiments minocycline was not expected to show any inhibitory effects

Fig. 1. Dose response effect (toxicity) of lysosomotropic agents on primary astrocytes. Primary human astrocytes (HFA) were seeded in 48 wells tissue culture plate and tested for toxicity of Bafilomycin-A and chloroquine in a dose dependent manner. The toxicity was monitored by quantitative cell viability assay system after 48 h in quadruplicate using nitro-propionic acid (8 nM 3) as a positive control.

Fig. 2. HIV infection of astrocytes with lysosomotropic agents. (A: a), HFA infected with HIV-NLENY1 showing infected astrocyte; (b), infected astrocytes in (a) with GFAP marker immunostaining; (c), control HFA under green filter; (d), panel (c) showing GFAP positivity; (e), showing HIV-infected HFA in the presence of 2.5 μM chloroquine (chloro, treatment for 2 h ) with a light phase view in (f); (g), HIV-NLENY1-infected lymphocytic cells showing trans-infection in astrocytes upon co-culture, infected (fluorescent) small round lymphocytes near leaf like infected astrocyte (green) on 8 days post co-culture; (h) control HFA co-cultured with normal lymphocytes; (i) Jurkat cells infected with NLENV3Y1 (6 dpi); (j) uninfected Jurkat cells as controls; (k) HFA infected with VSV-NLENY1 virus 4 dpi and (l) uninfected control HFA cells. (B) HIV-NLENY1 infection of astrocytes in the presence of chloroquine (chloro) and bafilomycin-A (Bafi, 100 nM) and minocycline (Mino, 25 μg/ml) (n=3): Lysosomotropic agents revealed several fold increase in HIV infection of astrocytes. Single infected astrocyte from chloroquine-untreated cultures is shown in insert. Minocycline treatment for 2 h serves as a negative control in parallel with chloroquine and bafilomycin or in combination. Minocycline does not affect endosomal pH or endocytosis. (C) Effect of lysosomotropic agents on NLENV3Y1 (hybrid R5, V3 region from YU-2) virus infection in HFA. NLENV3Y1 infection of astrocytes in the presence of chloroquine revealed 12–25 fold increase in HIV infection (compared to untreated infected astrocytes) in 27 days of follow-up (n=5).
on HIV infection of astrocytes in 2 h of treatment (either preinfection or post infection treatments). Indeed, minocycline treatment (25 μg/ml) alone or in combination with chloroquine (2.5 μM) did not show any interfering effect on chloroquine enhanced HIV infection in astrocytes (Fig. 2B). The peak infection in either untreated or chloroquine/bafilomycin-A treated HIV-infected astrocytes was seen between 10–15 days post infection (Figs. 2A and B) compared to lymphocytes (peak HIV infection at 6–8 days, Fig. 2A). In contrast, NLENVY1-(hybrid R5)-virus (brain derived R-tropic YU-2 strain V3-envelope region engineered in NLENV1 backbone deficient in its own V3 region) revealed incredibly weak infection in HFA; but, chloroquine treatment resulted in similar degree of increase in HIV infection as seen with NLENV1 (X4)-infected chloroquine-treated HFA (Fig. 2C), indicating endocytosis mediated viral entry (chloroquine prevented viral particles degradation in lysosomes) irrespective of viral envelope (CD4- and CXCR4-independent infection, manuscript in preparation). Besides, nonspecific effects such as release of residual virus without viral replication by astrocytes (Clarke et al., 2006) upon treatment with these agents, was ruled out by the cumulative evidence of rescuing of infectious virus from these chloroquine-treated HIV-infected astrocyte cultures by trans-infection of lymphocytes or vice versa (Fig. 2A-g, detailed manuscript in preparation), YFP-gene expression (Figs. 2A–C) and HIV genome integration (Fig. 2D). This indicated that low HIV infection in astrocytes is productive and through integrated viral DNA but not merely by unintegrated viral DNA. Hence, to corroborate further elevated viral infection and to implicate invigorating-synergism between HIV and chloroquine, we monitored the effect of chloroquine on either acutely VSV- or ba

NLENY1 (HIV pseudotyped with VSV-G envelope) infected or persistently infected astrocytes clonally obtained after infection. Neither chloroquine nor Bafilomycin-A, were able to inhibit wild type HIV replication in persistently infected astrocytes when compared to minocycline, instead enhanced p24 expression was observed (Fig. 3A). Similar to our observations, bafilomycin-A recently has been shown to elevate HIV infection in astrocytes (Deiva et al., 2006). Since long term treatment with minocycline revealed significant anti-HIV activity on persistently infected astrocytes, however, on combination with lysosomotropic agents could not attenuate the enhanced HIV replication by chloroquine or bafilomycin (Fig. 3A). In contrast, lysosomotropic agents revealed strong antiviral effect on acute VSV-pseudotyped HIV-infected astrocytes suggesting the inhibitory activity on pH-dependent viral endocytosis entry (VSV enters through pH dependent endocytosis) (manuscript in preparation). However, it is still ambiguous whether the presence of chloroquine during HIV infection of astrocytes has any additional effects other than lysosomotropic effect. Subsequently, these lysosomotropic agents in our further investigations did not show any stimulatory effect on the HIV-LTR promoter in latently HIV-infected monocytic (THP) p89GFP (dual tropic) cells reactivated with TNF-α. (A) Bafilomycin (100 nM) and chloroquine (2.5 μM) profoundly increased HIV replication (p24) in persistently infected astrocytic cells for 4 days (n=3). Further, long term treatment with minocycline revealed significant anti-HIV activity on persistently infected astrocytes, however, on combination with lysosomotropic agents could not attenuate the enhanced HIV replication by chloroquine or bafilomycin. (B) Effect of chloroquine and bafilomycin-A (same concentrations as shown above) on HIV-LTRGFP reporter cells: THP9 cells latently infected with pH9.GFP virus (dual tropic) were treated with chloroquine and bafilomycin-A for 48 h. Both the drugs failed to induce reactivation of latent HIV compared to TNF-α (positive control). Control cells did not reveal green fluorescence above background levels.

Our study unambiguously shows that chloroquine profoundly increases the permissivity of neuro-glial cells to HIV infection. In the pH-dependent pathway, the acidic environment of endosomes is used for release of viral nucleocapsids into the cytoplasm for viral replication. Therefore, lysosomotropic agents will block productive viral replication in a pH-dependent viral entry route. However, in a pH-independent viral entry route, viral replication will be unaffected by these agents. Albeit, when pH-independent viruses (such as HIV) enter through pH-dependent route via endocytosis in the absence of viral receptors, endocytosed viral particles will be flagged for degradation pathway (Fig. 4). However, the application of lysosomotropic agents may molecularly tune the astrocytes for efficient release of entrapped viral nucleocapsids from endosomes (without degradation) and subsequently lead to more number of viral-DNA copies integration. This will lead to enhanced productive HIV infection (Fig. 4) (Fackler and Peterlin, 2000; Wei et al., 2005; Chauhan et al., manuscript in preparation). Similarly, a study by Clarke et al. (2006) has demonstrated that astrocytes uptake sufficient number of HIV particles via phagocytosis/macropinocytosis without viral replication and subsequent lysosome-mediated degradation or release of undegraded viral particles to the extracellular environment by an unknown mechanism. This study however, could not demonstrate viral integration which possibly may be due to natural weak infection of astrocytes in contrast to present study wherein lysosomotropic agents have facilitated the rate of viral infection several fold (Figs. 2 and 3).

Hence, our observations led to a conjecture that chloroquine potently strengthens the endocytosis mediated productive HIV infection in non-CD4 positive astrocytes through reprogramming of endosomal pH, which otherwise without chloroquine would be minimally productive (Figs. 2–4). Inadvertently, chloroquine-mediated permissiveness of neuro-glial cells may have serious consequences in HIV infection of the brain, especially in endemic
areas with malarial co-infection where this drug is more commonly prescribed. In particular, chloroquine may promote early development of neurological complications and establishment of a stable long term reservoir of HIV in otherwise non-permissive long lived neuro-glial cells. Indeed, besides its impuissance in curtailing HIV infection of the brain, chloroquine could be useful in inhibition of other enveloped viral infections that have a pH-dependent entry route.

Materials and methods

Primary human fetal brain cell culture and cell lines

Primary human fetal astrocytes were cultured from human fetal tissues as described earlier (Chauhan et al., 2007). Human fetal brain specimens were obtained from fetuses of 8–14 weeks gestational age. Briefly, the brain tissue was mechanically dissociated in Opti-MEM with 5% heat inactivated fetal bovine serum (FBS) and antibiotic solution (penicillin G 10^5 U/ml, streptomycin 10 mg/ml, and amphotericin B 25 μg/ml). The dissociated cells (without trypsin) were cultured either in Opti MEM (0.2% N2 supplement,) for neurons or for astrocytes in Dulbecco’s modified Eagle medium (DMEM) with 10% FBS and antibiotics for at least 1 month prior to use in experiments. Further, after 8 weeks in culture, HFA after 3rd or 4th passage were used in experiments. The cultures were verified for purity using GFAP, MAP-2 and CD68 markers and no contamination of microglia but rarely few neurons were observed. SVGA (a human astrocytic cells sub-clone of the SVG cells), were maintained in DMEM supplemented with 2 mM L-glutamine, 10% FBS, and antibiotic solution. Jurkat and 293T cells were grown in RPMI-1640 supplemented with 10% FBS. The astrocytes viability in lysosomotropic agents (chloroquine and bafilomycin-A) was monitored by Cell Quant-Blue Cell viability assay kit (BioAssay Systems, Hayward, CA), the cells after treatments with lysosomotropic agents were incubated with the dye for 3 h and read in a spectrophotometer (Molecular devices) using 530 nM excitation and 590 nM emission filters and relative fluorescent units were plotted.

Viral constructs and plasmids

The green reporter HIV-proviral DNA construct was created by inserting YFP gene between viral envelope and nef genes without impairing the HIV open reading frames as reported earlier (Kutsch et al., 2002). VSV-G expression vector; HIV-proviral DNA pNL4-3 and pNLE-R+ proviral infectious DNA were obtained from NIAIDS reagent facility (NIH, USA).

Virus infection

Primary human fetal astrocytes (HFA), lymphocytic cells (Jurkat) and astrocytic cells (SVGA) were used in infection studies. NL-4–3, NL-EN-Y1, NLENV3Y1 and VSV-pseudotyped HIV viral stocks were prepared by transfection of 293T cells using 10–15 μg of plasmids in 100 mm dishes. Virus inoculum containing from 30–1000 ng/ml was used for infection of lymphocytes and astrocytes. Treatment of HIV-infected HFA with lysosomotropic agents was performed in 6 well tissue culture plates with 50,000–75,000 astrocytes (HFA) in each well and on third day post culture treated with either chloroquine or bafilomycin-A, for 2 h (2.5 μM) and then infected with HIV for 2 h. Inoculum was washed 2 times with medium and chloroquine added again from 4 h to 4 days, and cultures were followed up for 2–3 weeks by monitoring green fluorescence and p24 levels by ELISA (ZeptoMetrix Corp., NY, USA). Data from experiments were expressed as mean±SEM. Multiple infection and transfection experiments (more than 5 times) were performed to draw the inference. Latent viral reactivation studies were performed on monocytic THP89CFP cells with chloroquine and bafilomycin-A using TNF-α as a positive and minocycline (only 2 h treatment) as negative controls.

Fig. 4. Diagrammatic representation of natural or conditional (under the influence of lysosomotropic agents) HIV life cycle in human astrocytes: (1) Natural HIV entry route in astrocytes (in the absence of CD4-receptor) via endocytosis is least productive as viral particles may be degraded in the endosomes. Possibly few intact viral nucleocapsids will be released from the endosomes and would establish minimum infection. (2) Induced or conditional HIV entry route under the influence of lysosomotropic agents (chloroquine or Bafilomycin-A): Lysosomotropic agents will reprogram the endosomal pH and would result in release of more viral nucleocapsids. The higher number of released viral nucleocapsids will lead to more events of viral-DNA integration and subsequently more productive HIV infection.
Viral integration

Viral integration in HIV-infected astrocytes was monitored by Alu-HIV-LTR PCR (Clarke et al., 2006). The following primer sequences were used: First round of Alu-PCR was performed by primers INT-1, 5′-TGCTGGATTACGG GCCTGAG-3′ and INT-2, 5′-TAGACCAGATC-TGACGTTGGG-3′ using following program: 96 °C/3 min [96 °C/45 s, 60 °C/15 s, 72 °C/59 s] 35 cycles and extension 72 °C/7 min. In the second round 5 μl products from first amplification were amplified using primers INT-N1, 5′-GGCTAACTGGAAAC-CCACTG-3′ and INT-N9, 5′-CTGTAGAGATTTTCCACGTG-3′ with the following cycle program: 96 °C/3 min [96 °C/45 s, 60 °C/15 s, 72 °C/45 s] 35 cycles and extension 72 °C/7 min.

Immunofluorescence

The infected astrocytes were fixed in 2% paraformaldehyde (PBS) for 15 min and permeabilized with 0.2% Triton-X-100 (PBS) for 11 min followed by blocking with 5% BSA (PBS) for 1 h at room temperature (25 °C) (Chauhan et al., 2003). The treated cells were overlaid with primary antibodies at optimum working dilutions (anti GFAP and anti MAP-2 at 1/500 dilutions, Sigma, St Louis, USA) in a moist chamber at 25 °C. Subsequently, the specimens were washed four times with PBS, stained with DAPI (PBS) during the last washing for 10 min and finally rinsed with PBS and mounted with anti fading agent (biomeda, CA, USA). The stained specimens were stored at 4 °C or 25 °C until examined. After washing 5 μl products from first amplification were amplified using primers INT-N1, 5′-GGCTAACTGGAAAC-CCACTG-3′ and INT-N9, 5′-CTGTAGAGATTTTCCACGTG-3′ with the following cycle program: 96 °C/3 min [96 °C/45 s, 60 °C/15 s, 72 °C/45 s] 35 cycles and extension 72 °C/7 min.

Acknowledgments

We thank Drs. D. Levy (New York University) for NLENY1 and NLENV3Y1 constructs and J. Patton (University of South Carolina) for the discussions. This study was funded by NIH-grants R01NS050064 to AC and P01MH070306 to AN.