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Original Communication

Defective iron homeostasis in human immunodeficiency virus type-1 latency

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ABSTRACT

Highly active antiretroviral therapy has significantly improved the life of HIV-1-infected individuals, yet complete eradication of HIV-1 reservoirs (i.e., latently infected cells) remains a major challenge. We have previously shown that induction of the endogenous cytoprotective enzyme heme oxygenase-1 (HO-1) by its natural substrate hemin reduces susceptibility of T cells and macrophages to HIV-1 infection. In the present study, we demonstrate that hemin treatment stimulated virus production by latently infected ACH-2 cells, followed by cellular toxicity and death when stimulated with TNF-α or co-cultured with monocyte-derived macrophages (MDM). This cytotoxicity was associated with low levels of the iron-binding protein ferritin and the iron transporter ferroportin with lack of hemoglobin catabolic enzyme HO-1, resulting in substantial iron accumulation in the activated ACH-2 cells. Defective iron homeostasis in ACH-2 cells provides a model system for selective targeting of the latent HIV-1 reservoir by hemin-induced iron toxicity.

KEYWORDS: HIV-1 latency, macrophages, ACH-2, TNF-α, heme oxygenase-1

ABBREVIATIONS

MDM or Mϕ, monocyte-derived macrophages; HO-1, heme oxygenase-1.

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1. INTRODUCTION

Human Immunodeficiency Virus type 1 (HIV-1), the causative agent of the acquired immunodeficiency syndrome (AIDS), has caused a devastating pandemic affecting millions of people worldwide. Although combination antiretroviral therapy (cART) has significantly reduced AIDS-associated morbidity and mortality, these antiretroviral drugs do not fully restore health or normal immune status in HIV-1 infected individuals. The inability of cART to target latently HIV-infected cells warrants a search for new strategies towards a successful cure of HIV disease.

The life cycle of HIV-1 is linked to the activation state of its host cells and a number of host factors for entry and viral gene expression. Latent HIV in resting CD4-positive memory T cells can slowly replicate and induce new rounds of infection [1-4]. Interruption of cART therapy results in strong rebound of virus replication, even in HIV patients with undetectable viremia [5], which currently necessitates life-long treatment. Although several anti-latency apoptotic approaches have been attempted, eradication strategies have not achieved much clinical success. Over the past 10 years, our laboratory has developed cellular and pharmacological approaches to unravel how physiological manipulation of innate host response can provide cellular protection against HIV and other pathogenic infections [6-11]. We discovered that hemin induction of endogenous HO-1 in T-cells and macrophages dramatically reduces their susceptibility to HIV and other pathogens.

Since our 2006 publication [6], HO-1-dependent host protection has been confirmed by other investigators for numerous pathogens, including hepatitis B virus, hepatitis C virus, malaria, and recently Ebola [12-17].

Our previous studies established a link between hemin-induced heme oxygenase-1 (HO-1) and protection of primary T-cells and macrophages from HIV and other pathogenic infections; they strongly support the concept of activating a therapeutic, inducible innate host defense response. Widely distributed in tissues, HO-1 is an attractive therapeutic candidate against acute and latent HIV infection. In the present study, we demonstrate that hemin treatment of latently infected ACH-2 cells upon activation with either macrophages or soluble TNF-α accumulate substantial levels of intracellular iron and undergo dramatic cytotoxicity and death.

2. MATERIALS AND METHODS

Reagents

The FDA-approved drug Panhematin® was purchased from Lundbeck (manufactured by APP Pharmaceuticals). Mouse-anti-human HO-1 antibody was purchased from Enzo Life Sciences (Farmingdale, NY). The HIV-IIIB strain was purchased from Advanced Biotechnologies, Inc. (Columbia, MD).

Isolation and culture of monocyte-derived macrophages (Mϕ)

Monocytes were isolated from peripheral blood mononuclear cells of donors seronegative for HIV-1 and hepatitis B after leukophoresis and were purified by countercurrent centrifugal elutriation [18]. Cell suspensions contained >95% monocytes by criteria of cell morphology in Wright-stained cytosmears, granular peroxidase, and nonspecific esterase. The cells were cultured for 5 days in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS), 20 μg/ml gentamicin, and 1000 U/ml macrophage-colony stimulating factor. After 5 days, culture medium was completely removed, and the cells were washed three times with phosphate buffer saline (PBS), pH 7.4, and transferred to RPMI 1640 medium supplemented with 10% FBS and 20 μg/ml gentamicin. The resultant Mϕ were cultured for 2 additional days without M-CSF.

Culture of chronically infected ACH-2 cells

ACH-2 cells, a subclone of HIV-1 infected A3.01 cells, were cultured in RPMI 1640 supplemented with 10% FBS and 20 μg/ml gentamicin at 37 °C in 5% CO_2.

Coculture of Mϕ and ACH-2 cells

Mϕ (1 x 10^6) and ACH-2 cells (1 x 10^5) were cocultured in 24-well plates in 2 ml of RPMI 1640 medium supplemented with 10% FBS and 20 μg/ml gentamicin.

Quantitation of HIV-1 replication

Culture supernatants were assayed for HIV-p24 using an NEN/DuPont ELISA analysis kit (PerkinElmer Life Sciences, Boston, MA) according to the manufacturer's instructions. Assays were performed in triplicate.

Iron measurement

Intracellular levels of iron were determined using an iron assay kit (Sigma-Aldrich) according to the manufacturer's instructions. Assays were performed in triplicate.

Flow cytometry

Mϕ (1 x 10^6) were cocultured with ACH-2 for 48 hours in the absence or presence of 100 μM hemin. Cells were then harvested and washed three times with cold Hanks Balanced Salt Solution (HBSS). After centrifugation, the cell pellets were fixed in 4% paraformaldehyde for subsequent flow cytometric analysis.

Protein extraction and Western blot analysis

HO-1 induction in MDM was determined by Western blot (Enzo Life Sciences, Farmingdale, NY). Briefly, total cell protein extracts were prepared in modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 1% NP-40, 0.25% deoxycholic acid, 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, and 1 mM sodium fluoride) containing inhibitors of proteases (Roche Applied Science) and phosphatases (Sigma). After quantification using the Pierce BCA protein assay, total protein (2.5 to 5 μg per lane) was resolved on

a 12% Tris-glycine sodium dodecyl sulfate/ polyacrylamide gel electrophoresis (SDS/PAGE) gel. After transfer to a polyvinylidene difluoride membrane (Millipore), HO-1 and actin were detected using 1:5000 dilutions of primary rabbit polyclonal antibodies, followed by a horseradish peroxidase-conjugated anti-rabbit IgG. Protein bands were visualized using the ECL® (enhanced chemiluminescence) detection system (GE Healthcare). Western blots were performed by Kendrick Labs, Inc. (Madison, WI).

3. RESULTS AND DISCUSSION

In the present study, we used the Food and Drug Administration (FDA)-approved (for porphyria) pharmaceutical formulation of hemin, an inducer

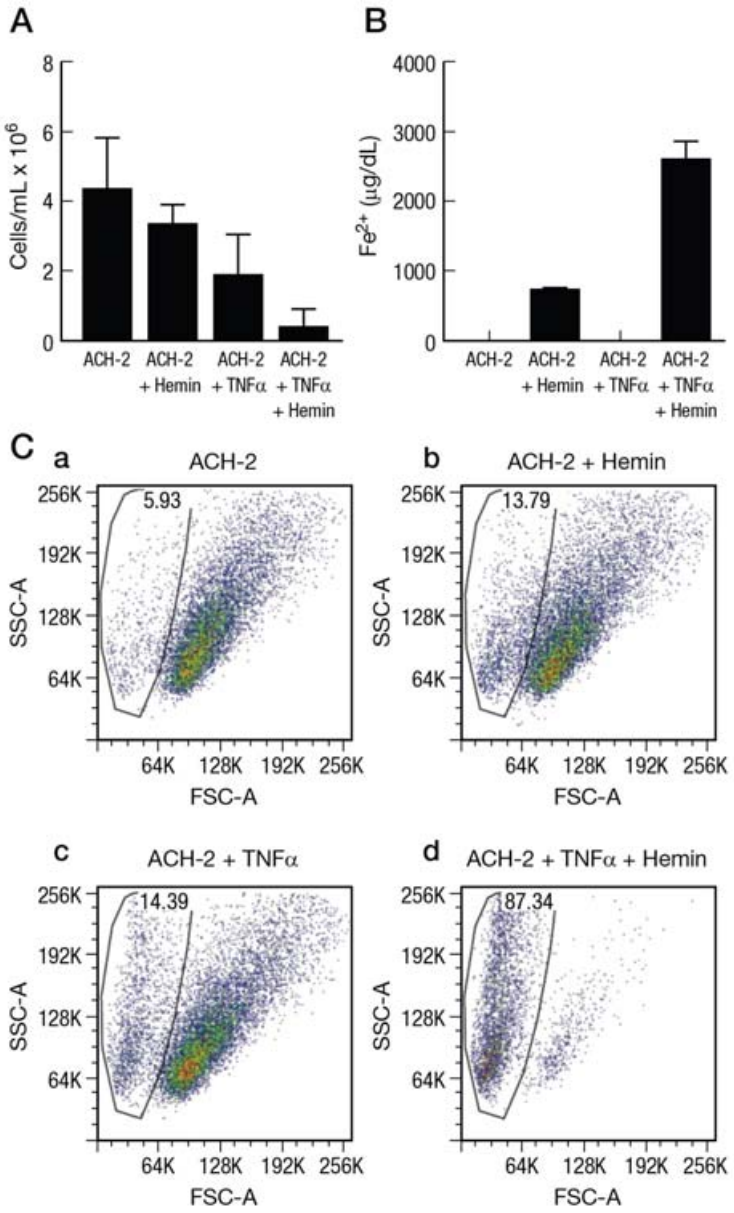

Fig. 1. Hemin induces intracellular iron-associated cytotoxicity in ACH-2 cells cultured in the absence or particularly in the presence of TNF-α. **A.** Trypan blue-negative cells (cells/ml). **B.** Intracellular Fe^{2+} expressed as µg/dL. **C.** Flow cytometric analysis of ACH-2 cells demonstrating induction of cell death.

of HO-1, and ACH-2 cells as a well-accepted model system for latent HIV-1 infection. We first examined effects of hemin on ACH-2 cell viability. We incubated ACH-2 cells in media containing 100 μM hemin in the absence or presence of TNF-α and quantified viable cells after 10 days. As shown in fig. 1A, hemin treatment was largely non-toxic to ACH-2 cells in the absence of TNF-α, but in its presence induced dramatic (>80%) cellular toxicity and death (Fig. 1A).

Our physiological rationale for adding TNF-α was: (a) TNF-α and other pro-inflammatory cytokines are secreted in established HIV infection [19-21]; and (b) elevated levels of these cytokines directly correlate with viral load during progression of HIV-1 infection, with NF-κB activation [22]. Our findings suggest that latently infected cells in HIV patients stimulated with endogenous pro-inflammatory cytokines might undergo similar cell death upon HO-1 induction.

Treatment of ACH-2 cells with hemin plus TNF-α resulted in five-fold elevated iron accumulation (Fig. 1B), but not with hemin or TNF-α alone. Because excess intracellular iron is cytotoxic, we hypothesized that it contributes to the toxicity in activated ACH-2 cells. Hemin-induced cytotoxicity in ACH-2 cells was further confirmed by flow cytometry. Combined forward scatter (FSC) and side scatter (SSC) analysis identified cell populations gated for live and dead cells. The FSC versus SSC plots shown in fig. 1C demonstrate that the great majority of ACH-2 cells cultured for 10 days with 100 μM hemin or 20 ng/ml TNF-α alone were viable (panels B, C). In striking contrast, ACH-2 cells cultured with both TNF-α and hemin exhibited greater than 80% cell death (panel D), confirming cellular toxicity.

Although activation of latently infected cells will induce a new round of infection, hemin treatment is known to completely block susceptibility of peripheral blood lymphocytes (PBL) and MDM to new HIV infection without significant toxicity [6]. PBL challenged with HIV-IIIB were productively infected with the virus on day 7, but in the presence of hemin, HIV-p24 was practically undetectable in culture supernatants (Fig. 2A). Treatment of latently infected ACH-2 cells with hemin produced weak activation of HIV-1 (Fig. 2B, C). This finding is partially consistent with a recent report

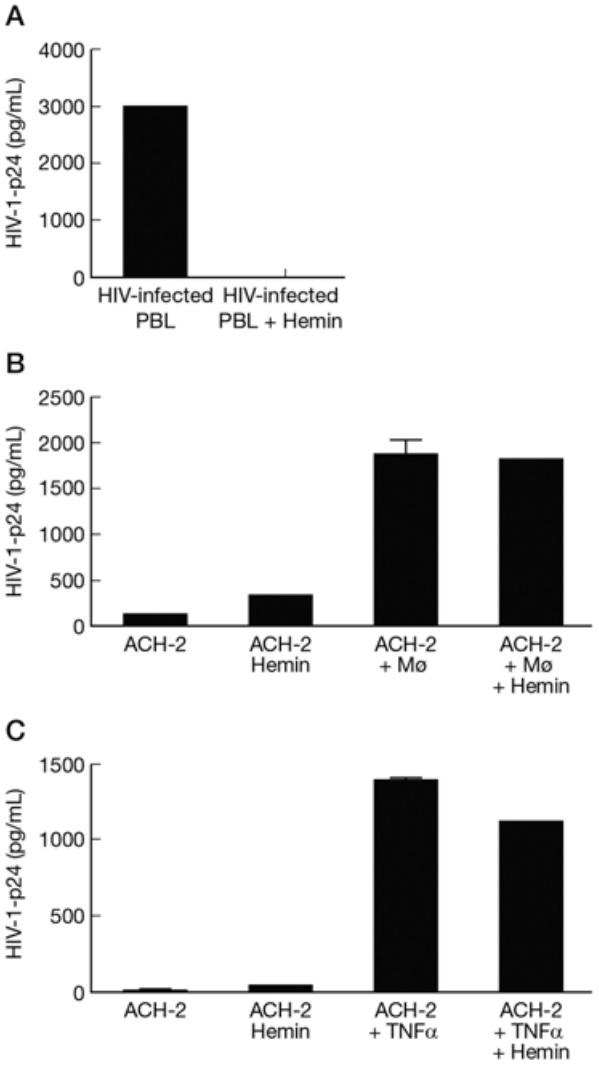

Fig. 2. A. Cell-free HIV-1-p24 levels in HIV-infected PBL on day 10. **B.** HIV-1-p24 in ACH-2 and ACH-2/ macrophage cocultures for 48 hours with or without 100 μM hemin. **C.** HIV-1-p24 in ACH-2 cells cultured 48 hours with or without TNF-α, plus 100 μM hemin.

using heme arginate [23], but we did not observe HO-1 induction in hemin-treated ACH-2 cells (Fig. 3A). In contrast, we found a ten-fold induction of HIV production in ACH-2 cells either cocultured with macrophages or stimulated with exogenous TNF-α (Fig. 2B, C) with or without hemin. Importantly, a substantial proportion of such activated ACH-2 cells were killed with hemin as shown in fig. 1. Therefore, this triple action of: (a) activation of virus replication; (b) induction of direct cellular toxicity in ACH-2 cells; and

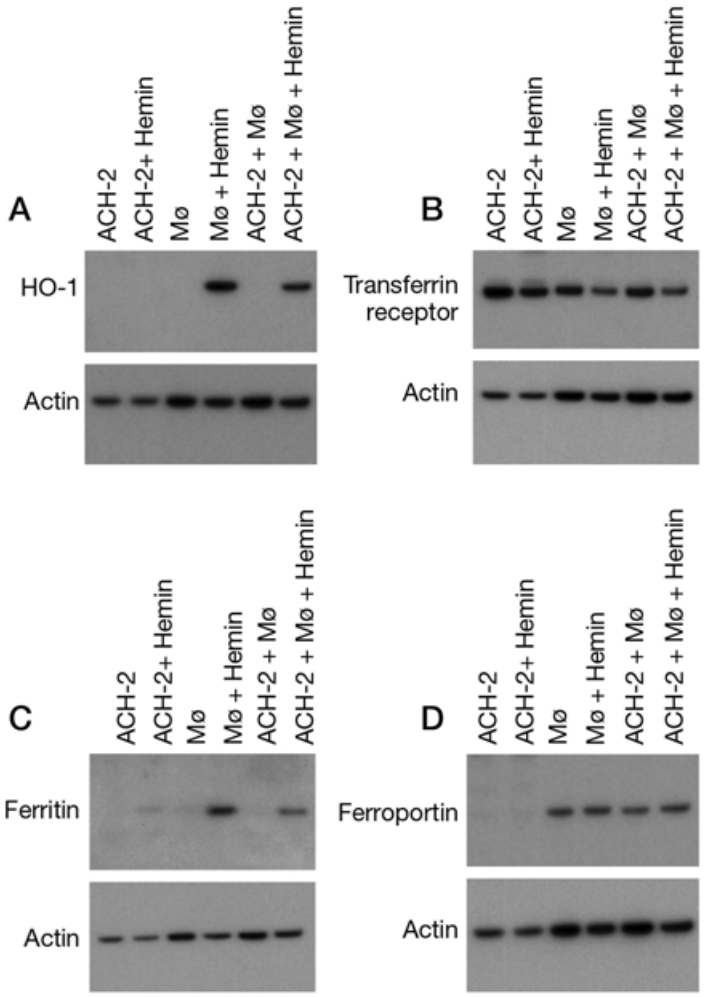

Fig. 3. Western blot analysis of total protein isolated from ACH-2 and ACH-2/macrophage cocultures for 48 hours in the absence or presence of 100 μM hemin. **A.** HO-1 expression. **B.** Transferrin receptor expression. **C.** Ferritin expression. **D.** Ferroportin expression. All blots were stripped and re-probed for actin as a loading control.

(c) stimulation of host resistance in uninfected cells to acute HIV infection, suggests that hemin treatment could provide an attractive new strategy for developing novel therapeutic interventions against HIV latency and infectivity.

Although hemin, TNF-α, and macrophages are normally non-toxic, the striking elevation of intracellular Fe^{2+} after treatment of latently infected ACH-2 cells suggested altered iron metabolism. We next examined whether altered levels of iron-transporting proteins could contribute to the elevated intracellular iron. We found that the transferrin receptor was expressed in all cell types and remained unchanged by hemin treatment (Fig. 3B). In contrast, latently infected ACH-2 cells expressed relatively low levels of the iron-binding protein ferritin and iron-exporting ferroportin than macrophages (Figs. 3C, D). Under normal physiological conditions, to avoid iron overload, cells shelter iron in ferritin. The low levels of ferritin and ferroportin with low HO-1 in hemin-treated ACH-2 cells could impair intracellular trafficking of ferroportin, resulting in reduced iron export. These results reveal higher iron retention and cytotoxicity of cocultured or cytokine-stimulated ACH-2 cells after hemin treatment. The low level of ferroportin may correlate with the clinical manifestations of ferroportin dysregulation [24, 25].

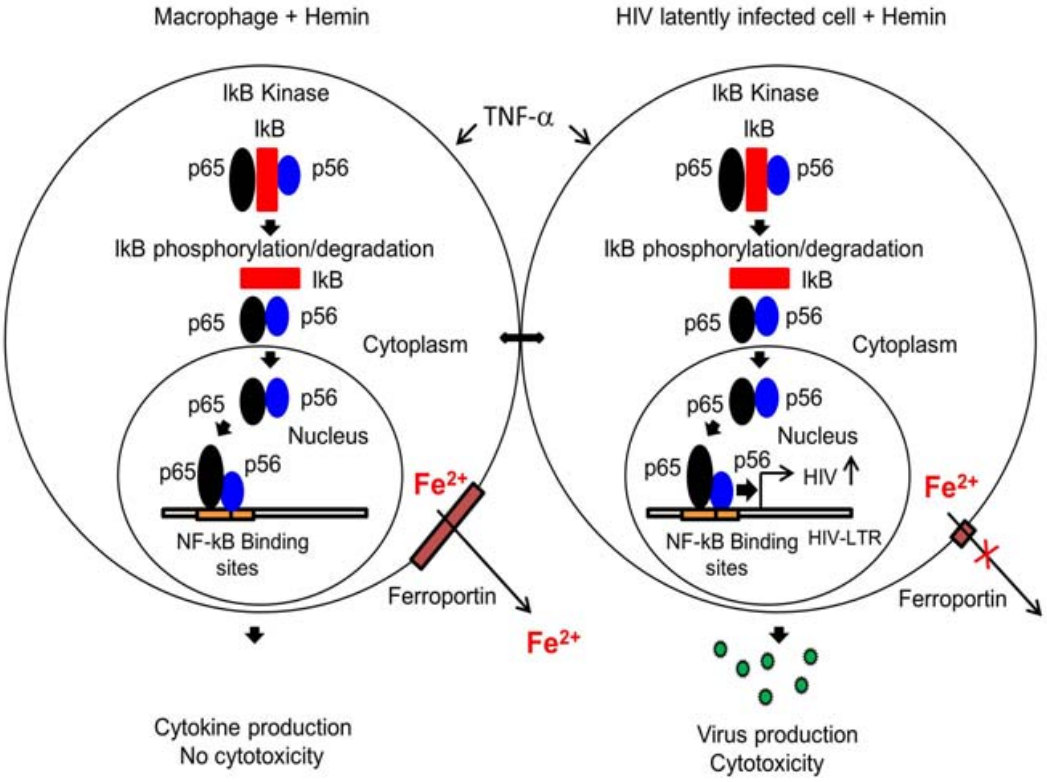

Fig. 4. Model for hemin-induced cytotoxicity in HIV-1 latently infected cells activated for virus production by TNF-α or macrophage coculture.

Fig. 4 presents a speculative model for the increased virus production and cellular toxicity in HIV-1 latently infected cells. Close proximity of HIV-latently infected cells to circulating or resident macrophages might account for their known activation to produce low levels of HIV virus. Such cell-to-cell contacts may also explain the observed high levels of endogenous TNF-α and other pro-inflammatory cytokines [21]. We discovered that HIV latently-infected ACH-2 cells undergo dramatic cell death after hemin treatment combined with TNF-α or macrophage coculture. This cytotoxicity is associated with dysregulation of Fe^{2+} and two important iron regulatory proteins.

4. CONCLUSION

In summary, we have demonstrated how hemin can disrupt pathogenesis through host defense of uninfected cells against HIV-1 infection, while simultaneously inducing striking cytotoxicity in activated HIV-latently infected cells. We suggest that the hemin-induced cellular toxicity of virus-producing activated ACH-2 is associated with compromised iron metabolism and resultant cell death. Our findings suggest a potential opportunity for clinical application of hemin in treating HIV-1 infection and eradicating viral latency, especially because hemin is already FDA-approved for treatment of a different disease.

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AUTHOR CONTRIBUTIONS

S. D. conceptualized, designed, performed experiments, analyzed data and wrote the paper;

H. H, Z. Z, and R. A. performed experiments and analyzed data; K. M. Y. analyzed data.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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