Effects of HIV-1 gp120 and tat on endothelial cell senescence and senescence-associated microRNAs

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Abstract
The aim of this study was to determine, in vitro, the effects of X4 and R5 HIV-1 gp120 and Tat on: (1) endothelial cell senescence and (2) endothelial cell microRNA (miR) expression. Endothelial cells were treated with media without and with: R5 gp120 (100 ng/mL), X4 gp120 (100 ng/mL), or Tat (500 ng/mL) for 24 h and stained for senescence-associated β-galactosidase (SA-β-gal). Cell expression of miR-34a, miR-217, and miR-146a was determined by RT-PCR. X4 gp120 significantly increased cell expression (~100%) versus control. X4 gp120 significantly increased miR-34a (1.60 ± 0.04 fold) and miR-217 (1.52 ± 0.18), but not miR-146a (1.25 ± 0.32). R5 gp120 significantly increased miR-34a (1.60 ± 0.40 fold) and decreased miR-217 (1.52 ± 0.18), but not miR-146a (1.25 ± 0.32). R5 gp120 significantly increased miR-34a (1.23 ± 0.07) and decreased miR-146a (0.56 ± 0.07). Tat significantly increased miR-34a (1.49 ± 0.16) and decreased miR-146a (0.55 ± 0.23). R5 and Tat had no effect on miR-217 (1.05 ± 0.13 and 1.06 ± 0.24, respectively). HIV-1 gp120 (X4 and R5) and Tat promote endothelial cell senescence and dysregulation of senescence-associated miRs.

Introduction
Human immunodeficiency virus (HIV)-1 infection is associated with an increased risk and prevalence of atherosclerotic cardiovascular disease (CVD) (Islam et al. 2012; Gibellini et al. 2013; Wang et al. 2015). A major factor underlying the increased CVD burden with HIV-1 infection is endothelial damage and dysfunction (Subramanian et al. 2012; Gibellini et al. 2013). Although the mechanisms underlying the HIV-1-related vasculopathy are not fully understood, HIV-1-associated viral proteins are known to have deleterious effects on the endothelium. Indeed, HIV-1 gp120 and transactivator of transcription (Tat) have been shown to cause endothelial dysfunction and, in turn, have been linked to CVD pathogenesis (Kline and Sutliff 2008; Wang et al. 2015; Haser and Sumpio 2017). For example, HIV-1 gp120 induces endothelial cell apoptosis and impairs endothelial vasodilatory capacity (Ullrich et al. 2000; Jiang et al. 2010; MacEneaney et al. 2011) and, HIV-1 Tat promotes endothelial cell activation and enhances the development of atherosclerotic lesions (Rusnati and Presta 2002; Duan et al. 2013).

Endothelial cellular senescence is both a cause and consequence of endothelial dysfunction and atherosclerosis (Vasile et al. 2001; Minamino and Komuro 2007; Erusalimsky 2009). Senescent cells cease to perform functions necessary to maintain vascular homeostasis and, in turn, have been linked to CVD pathogenesis (Kline and Sutliff 2008; Wang et al. 2015; Haser and Sumpio 2017). For example, HIV-1 gp120 induces endothelial cell apoptosis and impairs endothelial vasodilatory capacity (Ullrich et al. 2000; Jiang et al. 2010; MacEneaney et al. 2011) and, HIV-1 Tat promotes endothelial cell activation and enhances the development of atherosclerotic lesions (Rusnati and Presta 2002; Duan et al. 2013).
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100 mL of PBS was bubbled with compressed N2 for 20 min followed by the addition of 15 mg of DTT and 100 mg of BSA and cooled on ice. Thereafter, 250 μL of the PBS solution was used to dissolve the Tat. The gp120s were diluted in culture media to the desired concentrations.

Cell culture

Human aortic endothelial cells (HAECs) were purchased from Life Technologies (ThermoFisher, Waltham, MA) and cultured in endothelial growth media (EBM-2 BulletKit)(Lonza, Basel, Switzerland) supplemented with the 100 U/mL penicillin and 100 μg/mL streptomycin under standard cell culture conditions (37°C and 5% CO2). Growth media were replaced 24 h after initial culture and every 2 days thereafter. Cells were serially passaged after reaching 80–90% confluence and cells were harvested for experimentation after reaching ~90% confluence on the 3rd passage. Cells were seeded into 6-well tissue culture plates (Falcon, Corning NY) and treated with media alone or media containing HIV-1 X4 gp120 (100 ng/mL), R5 gp120 (100 ng/mL) or Tat (500 ng/mL) for 24 h. After 24 h cells were stained with a senescence-associated β-galactosidase cytochemical stain or harvested for RNA isolation. Viral protein concentrations were similar to circulating levels in untreated HIV-1-seropositive adults (Oh et al. 1992).

Materials and Methods

Viral Proteins

Recombinant HIV-1 proteins Tat and Bal gp120 (R5) were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAD, NIH) and gp120 Lav (X4) was purchased from Protein Sciences Corporation (Meriden, CT). To reconstitute Tat, (Minamino and Komuro 2007). Concomitant with functional arrest, senescent endothelial cells develop a proinflammatory senescence-associated secretory phenotype resulting in the production and release of several cytokines and proinflammatory signaling molecules, such as IL-6 and TNF-α (Prattichizzo et al. 2016; Acosta et al. 2013). These and other senescence-associated cytokines can induce senescence in bystander endothelial cells and stimulate monocyte activation and infiltration through the vascular wall, promoting atherosclerotic lesion development (Acosta et al. 2013). Thus, endothelial cell senescence is regarded as a critical factor in the pathogenesis and progression of atherosclerotic vascular disease (Vasile et al. 2001; Minamino and Komuro 2007; Erusalimsky 2009). Accelerated senescence is a potential mechanism underlying the dysfunctional endothelial phenotype induced by HIV-1 gp120 and Tat. However, the impact of viral proteins on endothelial cell senescence is not well understood.

MicroRNAs (miRs) are short (~22 nucleotides), endogenous, single-stranded, noncoding RNAs that are involved in the regulation of a number of physiological and pathological processes (Kim 2005). miRs interact with mRNAs on the basis of complementary sequences between the miRNAs and the 3′-untranslated regions (3′UTRs) of the target mRNAs resulting in downregulation of target gene expression posttranscriptionally by either mRNA degradation and/or by suppressing translation (Bartel 2004). It is now recognized that miRs, specifically miR-34a, miR-146a, and miR-217, play a pivotal role in regulating endothelial cell senescence (Bhaumik et al. 2009; Menghini et al. 2009; Ito et al. 2010; Badi et al. 2015). Altered expression of these senescence-associated miRs (SA-miRs) has been shown to mediate endothelial senescence under various physiologic and pathologic conditions (Menghini et al. 2013). The effect of HIV-1 viral proteins on the cellular expression of SA-miRs, however, is currently unknown.

Accordingly, the aim of this study was to determine: (1) the effects of X4 and R5 HIV-1 gp120 and Tat on endothelial cell senescence and (2) whether the cellular expression of SA-miRs (miR-34a, miR-146a, and miR-217) is adversely affected by these HIV-1 viral proteins.

Materials and Methods

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Intracellular miR-34a, miR-146a, and miR-217 Expression

RNA was isolated from cells (1.0 × 10^5) harvested from each treatment condition using the miRCURY RNA isolation kit (Exiqon, Vedbake, Denmark). Thereafter, isolated RNA concentration was determined using a Nanodrop Lite spectrophotometer (ThermoFisher, Waltham, MA) (Ye et al. 2013).

Immediately after RNA isolation, 150 ng of RNA was reverse transcribed using the miScript II Reverse Transcription Kit (Qiagen, Hilden, Germany). RT-PCR was performed using the BioRad CFX96 Touch Real Time System along with the miScript SYBR green PCR kit (Qiagen) and specific primers for miR-34a, miR-146a, miR-217 (Qiagen) (Hao et al. 2014; Murphy et al. 2015; Shaker et al. 2015). All samples were assayed in duplicate. miRNA expression was quantified using the comparative Ct method and normalized to U6 (Ye et al. 2013). The fold change of each transcript was calculated as the \(2^{-\Delta\Delta Ct}\), where fold change (AU) = \(2^{-(Ct_{miR\ experimental}-Ct_{RNU6experimental}-Ct_{miR\ control}-Ct_{RNU6control})}\).

Statistical analysis

Differences between treatments were determined by analysis of variance. Where indicated by a significant F value, post hoc tests with Bonferroni correction for multiple comparisons were performed. Changes in relative expression of miRs to the viral proteins were determined by two-tailed, unpaired Student’s t-test. Data are reported as mean ± SEM for four independent HAEC experiments. Statistical significance was set a priori at \(P < 0.05\).

Results

Endothelial cell senescence in response to each viral protein is shown in Figure 1. The percentage of senescent cells was significantly in higher in cells treated with X4 gp120 (32 ± 1%), R5 gp120 (30 ± 3%), and Tat (30 ± 1%) proteins compared with control untreated cells (18 ± 2%). Moreover, the magnitude of increase in senescent cells was similar among the HIV-1 viral proteins. Senescence in cells treated with denatured (boiled and sonicated) viral proteins was not different from control (data not shown).

In response to X4 gp120 treatment, cellular expression of miR-34a (1.60 ± 0.04 fold) and miR-217 (1.52 ± 0.18 fold) significantly increased (~60% and 50%, respectively) compared with control. There was no significant effect of X4 gp120 on the cellular expression of miR-146a (1.25 ± 0.32 fold) (Fig. 2). In response to R5 gp120, cellular expression of miR-34a (1.23 ± 0.07 fold) was increased (~25%; \(P < 0.05\)) and cellular expression of miR-146a (0.56 ± 0.07 fold) was decreased (~80%; \(P < 0.05\)) compared with control. There was no significant effect of R5 gp120 on the cellular expression of miR-217 (1.05 ± 0.13 fold) (Fig. 3). Treatment with Tat resulted in a significant increase (~50%) in the cellular expression of miR-34a (1.49 ± 0.16 fold) and significant decrease (~80%) in the cellular expression of miR-146a (0.55 ± 0.23 fold) compared with control. Cellular expression of miR-217 was not significantly affected by Tat (1.06 ± 0.24 fold) (Fig. 4).

Discussion

The primary new findings of this study are as follows: (1) HIV-1 R5 and X4 gp120 and Tat markedly increase endothelial cell senescence; and (2) the endothelial expression signature of specific SA-miRs is adversely altered by these proteins, thereby promoting a more senescence prone cellular phenotype. To our knowledge, this is the first study to determine the direct effects of HIV-1 proteins on endothelial cell senescence and expression profile of SA-miRs.

Endothelial senescence initiates and promotes a number of phenotypic changes that renders the endothelium prone to atherosclerosis (Menghini et al. 2013). For example, in addition to the release of proinflammatory cytokines, nitric oxide production has been shown to be...
significantly reduced in senescent endothelial cells increasing the susceptibility of the endothelium to atherosclerosis and thrombosis (Sato et al. 1993; Hoffmann et al. 2001; Matsushita et al. 2001; Krouwer et al. 2012). Furthermore, senescence negatively affects the regenerative and angiogenic capacity of the endothelium diminishing its reparative capacity and promoting atherosclerotic lesion development (Vasile et al. 2001; Minamino et al. 2002; Chang et al. 2005; Minamino and Komuro 2007; Erusalimsy 2009). Indeed, senescent endothelial cells have been found in vivo at atherosclerotic sites in both the aorta and coronary arteries (Vasile et al. 2001; Minamino et al.)
2002). The results of this study demonstrate that HIV-1 gp120 and Tat proteins accelerate endothelial cell senescence. The percentage of SA-β-gal stained HAECs markedly increased after exposure to both X4 and R5 gp120 and Tat. In fact, the degree of endothelial senescence induced (~30%) by each viral protein was almost identical, demonstrating remarkably similar, independent detrimental prosenescent effects. Unfortunately, we did not assess the combined effects of these viral proteins on endothelial senescence, a condition more representative of the in vivo endothelial HIV-1 environment. It is possible that the synergistic effects of gp120 and Tat on endothelial senescence would be greater than the observed individual effects reported herein. However, future studies are needed to address this issue.

Cellular senescence is a highly conserved process that is tightly regulated by specific gene expression programs (Gorospe and Abdelmohsen 2011) and their associated miRNAs (Qin et al. 2012). In fact, aberrant expression of SA-miRs is now regarded as a central feature of a senescent endothelial phenotype (Qin et al. 2012; Menghini et al. 2013). In this study we demonstrate, for the first time, the effects of HIV-1 gp120 and Tat on endothelial expression of miR-34a, miR-217, and miR-146a. These well-established SA-miRs have been shown to play a pivotal role in regulating senescence (Menghini et al. 2009; Ito et al. 2010; Vasa-Nicotera et al. 2011). Both miR-34a and miR-217 promote, whereas miR-146a quells endothelial cell senescence (Qin et al. 2012; Menghini et al. 2013). miR-34a is highly expressed in endothelial cells and the degree of expression increases during cell senescence (Ito et al. 2010; Staszel et al. 2011; Menghini et al. 2013). miR-34a targets and downregulates sirtuin-1 (SIRT1), a major regulator of endothelial cell longevity and metabolic function (Potente and Dimmeler 2008; Ito et al. 2010; Zhao et al. 2010). SIRT1 is a class III histone deacetylase involved in the deacetylation of a variety of proteins, including NF-kB and PPAR-γ (Haigis and Guarnera 2006). SIRT1 also exerts regulatory influence on FOXO3 and p53 (Chung et al. 2010; Ito et al. 2010). Decreased expression of SIRT1 associated with overexpression of miR-34a triggers senescence in endothelial cells (Ito et al. 2010; Qin et al. 2012). A seminal finding of this study was that HIV-1 X4 and R5 gp120 as well as Tat increased endothelial expression of miR-34a. Our finding in HAECs that Tat induces endothelial senescence and increased expression of miR-34a compliment and extend the results of Zhan et al. (2016) who demonstrated increased miR-34a expression in senescent endothelial cells from HIV-1 Tat transgenic mice. Similar to miR-34a, miR-217 also induces endothelial senescence through inhibition of SIRT1 (Menghini et al. 2009). Interestingly, however, unlike miR-34a, miR-217 expression was not uniformly affected by gp120 and Tat. Exposure to X4, but not R5 gp120 or Tat, caused an increase (~50%) in cellular expression of miR-217. The mechanisms underlying the differential effects of these viral proteins on miR-217 expression require further study.

Contrary to the prosenescent action of miR-34a and miR-217, miR-146a inhibits senescence by targeting proteins in the BCL-2 protein family (e.g., BCL-w and Bax) and NADPH oxidase 4. NADPH oxidase 4 is the most predominant NADPH oxidase isoform in endothelial cells and a potent mediator of oxidative stress-related senescence (Vasa-Nicotera et al. 2011; Rippo et al. 2014). miR-146a also diminishes the senescence-associated secretory phenotype via targeting of IRAK1, a key upstream activator of the NF-κB signaling pathway and the associated inflammatory cytokine (e.g., IL-6, IL-8) milieu (Bhaumik et al. 2009; Rippo et al. 2014). Thus, reduced expression of miR-146a is a hallmark feature of senescent prone endothelial cells (Vasa-Nicotera et al. 2011; Menghini et al. 2013). In this study, R5 gp120 and Tat, but not X4 gp120, significantly reduced (~80%) the expression of miR-146a in endothelial cells. The expression profile of miR-146a in response to the different viral proteins was also not uniform. Nevertheless, taken together, a clear and distinct prosenescent cellular miR signature emerged in response to gp120 and Tat exposure that is consistent with their common senescent effects. While R5 gp120 and Tat did not affect the expression of the prosenescent miR-217, they each significantly reduced the expression of the antisenescent miR-146a; whereas X4 gp120 increased miR-217 expression, but had no effect on miR-146a.

A limitation of this study is that we neither manipulated the levels of miR-34a, miR-217 or miR-146a to counteract the viral protein effects nor did we assess the bioavailability of the miR target proteins in order to firmly establish the contribution of these SA-miRs to the observed increase in senescence. Furthermore, mechanisms by which HIV proteins may alter miR expression remain to be explored. Endothelial cells express CXCR4 (Gupta et al. 1998) and CCR5 (Jones et al. 2011) suggesting that gp120 could exert its effects through triggering these receptors. Alternatively, gp120 may bind other cell surface molecules, be internalized and exert its effects intracellularly. It is well established that Tat binds cell surface molecules and is also readily endocytosed (Madani et al. 2011). Importantly, Tat is found in the peripheral blood of individuals with virus suppression on antiretroviral therapy (Madani et al. 2011) suggesting that it may contribute to endothelial dysfunction not only in untreated individuals, but in patients receiving therapy as well. The effects of HIV-1 viral proteins on SA-miR expression may represent an important mechanism.
underlying the proatherogenic vascular effects of gp120 and Tat (Wang et al. 2015; Zhan et al. 2016; Haser and Sumpio 2017). Future studies are needed to elucidate how these HIV-1 proteins differentially disrupt cellular miR expression patterns and, in turn, compromise target proteins that regulate endothelial cell viability and function. In conclusion, the results of this study demonstrate that HIV-1 X4 and R5 gp120 and Tat induce endothelial cell senescence potentially through disruption of SA-miRs. The pro senescent effects of gp120 and Tat on endothelial cells may contribute to the profound endothelial dysfunction and increased risk of atherosclerotic vascular disease associated with HIV-1 infection.

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Conflicts of Interest

The authors have no conflicts of interest to disclose.

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