MICROPARTICLES AND microRNA: NOVEL MEDIATORS OF HIV-1 ENDOTHELIAL DYSFUNCTION

by

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ABSTRACT

Hijmans, Jamie Graham (Ph.D., Integrative Physiology)

Microparticles and microRNA: Novel Mediators of HIV-1

Endothelial Dysfunction

Dissertation directed by Professor Christopher A. DeSouza, Ph.D.

Microparticles and miRNA represent novel mediators and effectors of endothelial cell function and, in-turn, vascular health. MicroRNAs (miRNAs or miRs) are short (20-22 nucleotide) non-coding RNAs involved in the homeostatic regulation of ~60% of all gene expression. miRNAs are transferred and regulated by microparticles, small anucleoid extracellular vesicles. Alteration of cellular miRNA expression by microparticles has been mechanistically linked with the pathogenesis and progression of atherosclerotic cardiovascular disease (ASCVD). Human immunodeficiency virus (HIV)-1 infection is associated with an increased risk and prevalence of ASCVD due, in-part, to endothelial cell damage and dysfunction. Interestingly, endothelial cells are neither targets nor reservoirs of the virus. As such, the detrimental effects of HIV-1 on endothelial cell function are complex, multifactorial and poorly understood. There are currently no data regarding the direct or indirect effects of HIV-1 on microparticle and miRNA biology. Accordingly, the aims of this dissertation were to determine: 1) the effect of HIV-1 related proteins gp120 and Tat on endothelial cell senescence and expression of senescence-associated miRNA; 2) if HIV-1 gp120 and Tat stimulate the release of endothelial microparticles in vitro and if viral protein induced microparticles adversely affect endothelial cell function; and 3) whether circulating microparticles are elevated in HIV-1-seropositive adults. In addition, we determined the effects of microparticles from HIV-1-seropositive adults on endothelial cell activation, inflammation, oxidative stress, senescence and

apoptosis, in vitro. To address these aims, we conducted three independent, sequential studies integrating basic and clinical approaches to optimize the translational potential of this work. The seminal and novel findings of these studies are that: 1) HIV-1 gp120 and Tat accelerate cellular senescence due, in part, to dysregulation of senescence-specific miRNA; 2) HIV-1 gp120 and Tat induce microparticle release from endothelial cells and these microparticles confer pathologic effects on endothelial cells enhancing inflammation, oxidative stress, senescence and apoptosis as well as altering cellular miRNA expression; and 3) circulating levels of microparticles are higher in HIV-1-seropositive adults and these microparticles induce a proatherogenic endothelial phenotype.

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Finally, this dissertation is dedicated to the loving memory my mother, Jackie Hijmans, whose spirit, kindness and love of learning inspired me to endure the challenges of this process.

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LITERATURE REVIEW

CIRCULATING microRNA: BIOGENESIS, FUNCTION AND ROLE IN CARDIOVASCULAR PHYSIOLOGY

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INTRODUCTION

The sequencing and annotation of the human genome and transcriptome has provided several insights into the complexity of protein synthesis. A notable discovery was ~97% of all RNA transcribed is non-coding in nature meaning no protein is generated from the sequence [1]. The term non-coding RNA (ncRNA) is an umbrella term used to describe several different functional RNAs including: ribosomal RNA, transfer RNA, long non-coding RNA and microRNA [1]. MicroRNAs (miRNAs) comprise a large group of ncRNA approximately 21-22 nucleotides (nts) in length which function as post-transcriptional regulators of gene expression. Primarily, miRNAs reduce target gene expression post-transcriptionally through binding of the 3'-untranslated region (3'UTR), 5'-untranslated region (5'UTR) or coding sequence of the target messenger RNA (mRNA), thereby driving mRNA degradation or by physically inhibiting translation [2]. Rarely, miRNAs may also increase protein expression by interacting with the 5'UTR of the mRNA which, in-turn, enhances the of regulatory and transcription related proteins [3]. It has been suggested that miRNAs act as nature's phenotypic "dimmer switch" by fine-tuning protein translation. The influence of miRNA is not minute or subtle; it is estimated that miRNA regulate up to ~50% of all mammalian gene expression [4].

In 1993 Ambros and colleagues [5] discovered the first miRNA by identifying a 22-nt ncRNA in *Caenorhabditis elegans* (*C. elegans)* which bound lin-14 mRNA and interfered with expression though anti-sense RNA-RNA interaction. The term *miRNA* was first introduced in 2001 to describe this class of RNAs in *C. elegans,* and is now used to classify these RNAs in all animal and plant cells [4]. To date 35,828 miRNAs from 223 different species have been identified and published in the central miRNA sequence repository (mirbase.org) [6].

Since their discovery miRNAs have been the focus of multi-disciplinary scientific inquiry resulting in more than 50,000 peer-reviewed articles on the topic to date (pubmed.gov). It is well established that miRNAs are critical regulators of many physiological processes including: development, cellular proliferation, differentiation, growth, apoptosis, senescence, and metabolism [7,8]. Furthermore, dysregulation of miRNA expression has been linked with the etiology of several pathophysiological conditions including cardiovascular disease, cerebrovascular disease, renal disease, metabolic disease, and cancer [7,9–13]. Interestingly, the altered expression of several miRNAs in the blood has also been linked with cardiovascular disease [7,12]. Because of this miRNAs have garnered clinical interest as both candidate biomarkers of disease and therapeutic targets [14].

This review of literature will describe: 1) the biogenesis of miRNA; 2) mechanisms of miRNA mediated gene expression regulation; 3) packaging and transport of miRNA in the circulation; 4) the pathophysiology of coronary artery disease; and 5) the involvement of miRNA in coronary artery disease and its risk factors.

miRNA BIOGENESIS AND PROCESSING

Biogenesis of miRNA occurs in 3 distinct steps: transcription of a primary miRNA, cleavage into a precursor miRNA, and processing into a functional or "mature" miRNA, referred to simply as miRNA [15]. The next sections will cover the mechanisms of each step and regulation of these mechanisms. It is important to note that this review will focus on mammalian miRNA biogenesis, however, due to the highly conserved nature of these process this concepts may be applied to lower order lifeforms as well.

Transcription

miRNAs transcription is tightly regulated by various transcription factors and is known to be highly specific to certain cell types and developmental states [4]. miRNAs are predominantly transcribed by RNA polymerase II [16] with the exception of a subset located near genomic Alu repeats which are transcribed by RNA polymerase III [17]. The coding sequences for miRNA are either located within another gene (intragenic) or between other protein coding genes (intergenic) [18]. The majority of mammalian miRNAs are intragenic and organized as introns (intronic miRNA) of coding and non-coding "host gene" transcriptional units [19]. Although this is less common, intragenic miRNA may also be transcribed as exons (extronic miRNA) of coding or non-coding mRNA transcripts [19]. miRNA genes also are located intergenically as independent genes containing dedicated regulatory and promoter sequences. Importantly, intergenic and intragenic miRNA genes are often polycistronic coding for a "cluster" of miRNAs which may be regulated by one or multiple regulatory sequences [15,19].

The majority of intragenic miRNA transcription is dependent upon factors regulating the host gene [18]. However, host promotor activity is not required as transcription of 1/3 of intragenic miRNA is regulated independently of host gene promotors and regulatory sequences [15,19]. miRNA transcription may be regulated in a non-specific manner by "master regulator" transcription factors (TF) including C-Myb, NFY, MTF1, and AP2α [18]. Most transcription factors regulate a miRNA or a group of miRNAs, typically in a pathway dependent manner [2,18]. For example, the transcription factor p53 activates the expression of miR-34a which induces cell cycle arrest by regulating genes involved in multiple levels of that pathway [20,21]. It is known that miRNAs that target mRNAs from a common pathway are likely to be regulated and transcribed together [22]. For example, the transcription factor MITF regulates the expression of miR-199a and

miR-214 which initiates melanocyte development [18]. Transcription factors may act in a contradictory manner by positively regulating expression of a miRNA in one cell type and repressing expression in another. Of note, the transcription factor Myc stimulates the expression of oncogenic miRNA in cancer cells while inhibiting tumor suppressor miRNAs in other cell types [4].

Splicing and processing of miRNA transcripts frequently occurs co-transcriptionally [19]. Once transcribed the pri-miR transcript forms one or more hairpin or stem loop structure(s) within which the miRNA(s) is embedded [23]. The stem of the hairpin is 35±1 base-pairs (bp) in length, with a 10-11nt apical loop flanked by single-stranded RNA (ssRNA) tails on the 3' and 5' sides [23]. These transcripts are protected from degradation by the formation of a 5'-methyl guanylate (m7G) cap and a 3' poly(A)-tail similar to other RNAs [24]. Most pri-miRs contain common RNA sequences (motifs) at specific locations which allows for identification downstream. Figure 1 depicts the recently discovered primary sequence determinates of pri-miR stem loops: a 5'-UG-3' motif located at the base of the stem, a mismatched GNG (N=A, U, or C)

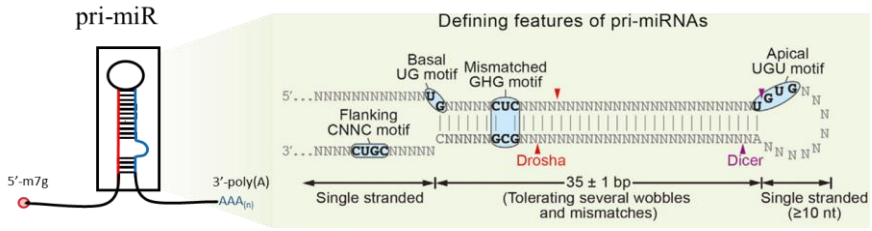

Figure 1: **Secondary structure and defining features of a pri-miR** with a 5'-methyl guanylate cap (5'-m7g) and a 3' poly(A)-tail. *Adapted from Fang and Bartel*(Fang and Bartel, 2015)

motif 7-nts from the 5' basal stem, an apical UGU motif and a CNNC motif in the 3' basal stem [25]. It appears that these structures are critical for proper pri-miR recognition and processing [25].

Nuclear Processing and Export

Mammalian pri-miRs are processed through one of two pathways; the canonical pathway or the non-canonical pathway. The vast majority of miRNAs are processed through the canonical pathway depicted in Figure 2 [26,27]. A small minority of miRNAs possess atypical transcriptional or structural characteristics which allow the miRNA to bypass a step in the canonical pathway [23].

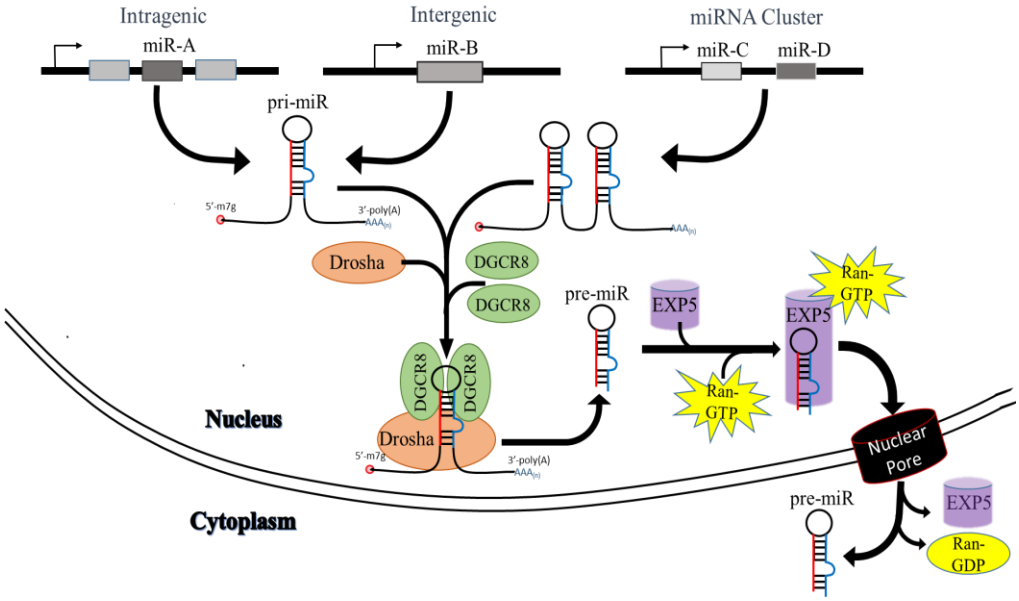

Figure 2: Schematic depicting miRNA transcription, nuclear processing, and cytosolic export. Primary miRNA (pri-miR) are transcribed from intragenic or intergenic genes as monocistronic (miR-A and miR-B) or polycistronic (miR-C and miR-D) transcripts. Transcripts are processed by Drosha along with Di Gorge Syndrome Critical Region 8 (DGCR8) proteins into precursor miRNA (pre-miR). Exportin-5 (EXP5) coupled with Ran-GTP export pre-miR into the cytoplasm and pre-miR are released from EXP5 upon Ran-GTP hydrolysis to Ran-GDP.

For the purposes of brevity and relevancy the canonical pathway is primarily discussed in this paper, and information on non-canonical processing is included where relevant.

Canonical processing begins with the cleavage of the pri-miR by the RNase III enzyme Drosha. The structural elements of the pri-miR mentioned above facilitate the binding of Drosha which is followed by binding of two DiGeorge syndrome critical region 8 (DGCR8) proteins

together forming the Drosha/DGCR8 holoenzyme referred to as the "microprocessor complex" [28]. The pri-miR is secured by the double stranded RNA (dsRNA) binding domains of Drosha and DGCR8, which aligns the substrate with the two tandem RNAse III domains of Drosha (RIIIDa and RIIIDb) for cleavage [28]. This results in the liberation of a ~65nt precursor miRNA (pre-miR) hairpin structure with a 2nt 3'-overhang [15,28]. Certain intronic miRNAs form shorter transcripts with a structure not recognized by the microprocessor complex [26]. These pri-miR are cleaved non-canonically by the spliceosome into pre-miRs that proceed through the rest of the canonical pathway [26].

Deletion of the Drosha gene results in a ~96% reduction in miRNA expression indicating the integral role Drosha plays in miRNA processing [29]. Microprocessor complex activity is known to be positively regulated RNA helicases p68 and p72, as well as SMAD and BRCA1proteins which stabilize Drosha [30]. Cell cycle proteins CDK6 and KRAS act as cofactors thereby increasing the affinity of the microprocessor complex for substrate, resulting in increased processing rate [19].

Nuclear export of the majority of pre-miRs is mediated by exportin-5 (EXP-5) which is involved in the nucleocytoplasmic transport of ncRNAs with similar stem-loop structures [31]. The current model posits that EXP-5 recognizes and binds substrates with a >14bp dsRNA stem, >5nt terminal loops and short 3' overhangs, characteristic of pre-miR [19,31]. EXP-5 along with Ran-GTP bind pre-miR and transport it through nuclear pore complexes to the cytosol where it is released upon Ran-GTP hydrolysis [17,31]. Interestingly, loss of EXP-5 leads to a reduction in miRNA expression, however, this reduction is modest compared to Drosha knockout, indicating EXP-5 is not essential for miRNA transport [29]. The compensatory nucleocytoplasmic transport mechanisms are unknown at this time [29].

Cytoplasmic Processing

Following nuclear export pre-miRs are processed by the RNAse III enzyme Dicer which generates a miR-miR* duplex (Figure 3) [32,33]. Dicer functional domains are strategically located to allow for highly specific endonuclease activity which is key for downstream processes. The PAZ domain of dicer is located at the N-terminus and preferentially binds the 2-nt 3'-ssRNA overhang created by Drosha [34].

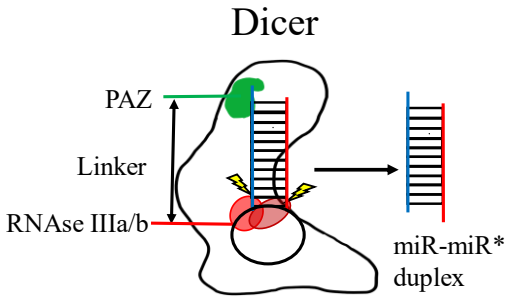

Figure 3. pre-miR cleavage by Dicer yielding a miR-miR* duplex. The pre-miR is oriented and sequred by the binding of the 3'-overhang by the PAZ domain. The linker region orients the pre-miR within the RNase IIIa/b domains for precise cleavage yielding a miR-miR* duplex.

The "body" of Dicer contains a linker region and the RNAse IIIa and RNase IIIb domains which contain the highly conserved active site of the enzyme [32,35]. The C-terminus or base of the protein contains a claw-like dsRNA binding domain (dsRBD) which is thought to clamp down on the pre-miR after binding [32,36]. The PAZ domain and linker region act as a "molecular ruler" such that RNase IIIa domains cleaves the 3'-arm sessile bond and the RNase IIIb cleaves the 5'-arm sessile bond a set number of nts from the end of the substrate [35]. Cleavage results in the loss of the pre-miR terminal loop and a portion of the stem resulting stem yielding a ~22-nt dsRNA molecule referred to as the miR-miR* duplex [32].

Dicer activity can be regulated by proteins and miRNAs. Human Dicer mRNA is targeted for repression by miRNAs of the let-7 family constituting a negative feedback loop [37]. Dicer is stabilized by accessory RNA binding proteins TRBP and PACT, in-turn, enhancing Dicer activity, but these proteins do not alter substrate specificity [36,38]. Depletion of TRBP and PACT leads to reduced miRNA production, however, Dicer is able to function without TRBP and PACT [39,40]. Another RNA binding protein, RBM3, binds pre-miRs promoting Dicer binding and enhances activity [41].

Formation of the RNA induced silencing complex

To become functionally active, a miRNA must be assembled into ribonucleoprotein complex termed the RNA induced silencing complex (miRISC). During this process the miR-miR* duplex is separated into the guide strand, which is retained, and passenger strand (miR*), which is degraded [42]. miRISC assembly occurs in three main steps: duplex loading, wedging and passenger strand ejection, which will be discussed in the following paragraphs.

The core component of the RISC is a protein from the Argonaut (Ago) family which exists in mammalian cells as four paralogs: Ago1-4 [3]. Each of the paralogs are known to have similar miRNA selectivity and functionally but onlyAgo2 possess endonuclease ("slicer") activity [43]. Ago proteins consist of four domains: PAZ, MID, N-terminal (N) and PIWI. The guide strand of the miR-miR* duplex is bound on the 3'-end by the Paz domain and the 5'-phosphate is bound in a specialized pocket between the MID and PIWI domains. [44] The N domain facilitates duplex unwinding [44].

The first step of RISC formation, duplex loading, is a dynamic process that requires ATP-hydrolysis and the aid of accessory proteins [42]. Several reports [2,3] indicate that duplex loading

requires a miRISC-loading complex consisting of Dicer, TRBP and PACT however, several carefully conducted studies demonstrate that the miRISC-loading complex is not required in mammals but may still aid in transfer [43–45]. Rather, chaperone proteins hsc70/hsp90 facilitate loading by binding Ago and hydrolyzing ATP to open, or activate, Ago such that Ago is able to bind the 5'-phospate on one end of the miR-miR* duplex [43,46]. Selection of the guide strand is based on the thermodynamic stability between base pairs located on the ends of the duplex with the 5'-strand of the less thermodynamically stable end being retained [45]. Kobayshi and colleagues speculate that the less thermodynamically stable end is preferred because it allows for faster separation of the guide and passenger strand [44].

In the second step, wedging, the N domain wedges open several base pairs at the 3' end of the guide strand and the PAZ domain binds to the now separated 3'- end of the guide strand [47]. The final step, passenger ejection, is mediated either by cleavage (slicing) and liberation of the passenger strand by Ago2, or by the unwinding and dissociation of the unbound passenger strand from the bound guide strand [42]. During wedging, the phosphate backbone of the guide miRNA helps to stabilize Ago which, in-turn, makes passenger strand ejection more thermodynamically favorable [47,48]. Passenger strand ejection rate is dependent upon miR-miR* duplex stability and unfavorable structures in the miRNA duplex including, mismatched based pairs that enhance the rate of passenger strand ejection [44].

It is commonly held that Ago proteins and miRNAs exist in the cell in a 1:1 ratio, however, recent evidence indicates that miRNAs are in a 7- to 13-fold excess, suggesting that Ago is limiting [49]. The importance of this stoichiometric ratio has yet to be determined. Hydroxylation of Pro 700 and phosphorylation of Ser 387 increases Ago recruitment to P-bodies which may enhance the processing of miRNAs present in P-Bodies [4]. Degradation of Ago is

controlled by two proteins, TRIM-NHL and TRIM71, which ubiquitinate Ago at different sites. Some believe that Ago is a principal regulator of miRNA activity, however, this notion is based on a handful of studies and more investigation is required to characterize the magnitude of Ago's influence.

MECHANISMS OF miRNA GENE EXPRESSION REGULATION

miRNA Binding

After passenger ejection, the affinity of miRNA for mRNA increases approximately 300 fold and the miRISC is now considered functional [48,50]. The majority of interactions between mRNA and the miRISC are mediated by Watson-Crick base paring of the miRNA's "seed sequence" or bases 2-8 on the 5'-end of the miRNA and a complementary sequence in the target mRNA [51]. Initially, it was believed that miRNA binding was mediated only through the seed sequence. Recent reports indicate that other regions of the guide miRNA interact with and bind mRNA as well [52]. Perfect Watson-Crick base-pairing between the miRNA and target is not only not required but represents the minority of interaction [52,53]. This enables a single miRNA to target several mRNAs and for a single mRNA to be target by several miRNAs [54]. It is important to note that the number and strength of base pair interactions between the miRNA and its target influences the biological outcome and is directly proportional to the magnitude of its effect [50].

RNA Induced Silencing Complex Mechanisms of Action

The binding of mRNA by the miRISC mediates either (1) the inhibition of translation and/or (2) mRNA degradation. Three groups have conducted large scale studies to characterize the predominate miRNA binding sites, each of which used slightly different methodology. The

first, published by Chi and colleges [55] determined that miRNAs predominately binds target mRNA in the 3'-UTR (40%) region followed by 25% in the 5'-UTR. Later studies conducted by Hafner *et al.,*[56] and Helwak *et al.,*[52] demonstrate the majority of miRNAs target mRNA 5'-UTR (42% and 43% respectively). miRNA-mRNA interactions are extremely dependent upon the miRNA and mRNA milieu present in the cells used for study and as such these findings serve as indications of the overall miRNA "iteractome" but cannot be used to characterize miRNA interactions in other cell types or phenotypes.

Regulation of gene expression by miRNA is thought to be mediated, in part, through the inhibition of translation. Translation may be inhibited by miRNA during ribosome assembly, initiation [57], elongation, or termination [58]. After binding to a target the miRISC complexes with the critical cofactor GW182, which allows the miRISC to mediate translational repression [59] primarily through the blockade of a critical helicase, eIF4A2, preventing translation [60]. Additionally, the miRISC:GW182 complex recruits other proteins which, together, block message translation, but this is currently not well understood [60–62].

Translational repression is usually followed by message degradation [61]. The miRISC will recruit poly(A)-tail binding and degradation proteins (Pan2/Pan3), which will deadenylate the mRNA [59,62]. Deadenylation may be followed by removal of the 5'-cap after which the mRNA is degraded through 5'-to-3' or 3'-to-5' exonucleolytic digestion [3,59,62].

Although several lines of evidence suggest that mRNA deadenylation followed by exonuclease digestion is the predominate form of message degradation,[63] a small portion of mRNA degradation is mediated by endonucleolytic cleavage of mRNA by Ago2, de-protecting the mRNA from exonuclease digestion [30]. This cleavage results from perfect complementary

binding between the seed sequence of the miRNA and mRNA or rare non-canonical interactions between miRNA and mRNA [64].

Degradation

Compared with biogenesis and mechanisms of action, miRNA degradation is poorly understood. An elegant study conducted by Marzi and others measured the decay rates to 200 different miRNAs *in vitro* and discovered that miRNA degradation rates are fairly heterogeneous [65]. Most miRNAs have half-lives of over 24 hours while a subset, including passenger strands, have significantly shorter half-lives of 4-14 hours [65]. Their findings suggest that degradation rate is directly proportional to miRNA:target stoichiometry for high affinity miRNA [65]. The author speculate that this is due to "target directed miRNA decay," or concomitant cleavage of miRNA and mRNA due to non-specific exonuclease activity [65]. This effect is not observed with low affinity miRNA because miRNA:mRNA interactions are fewer.

miRNA PACKAGING AND EXTRACELLULAR TRANSPORT

In 2008, several groups observed the presence of miRNA in cell-free plasma [66–68] which was followed by the observation of miRNA in every body fluid [69]. These observations were unexpected as synthetic, or naked, RNA is rapidly degraded in plasma due to the abundance of RNAse enzymes [70]. Plasma miRNA was found to be susceptible to degradation when RNAse was combined with proteinase K or a detergent indicating that miRNA are protected by lipids and/or proteins in the circulation [70,71]. Investigation into miRNA packaging revealed 3 classes of miRNA carriers 1) Ago proteins;[72] 2) lipid vesicles; and 3) high-density lipoprotein (HDL) [73,74]. It is estimated that 90-99% of miRNA present in circulation is bound to Ago proteins which are

thought to be passively released from cells as a result of membrane rupture[75] and actively exported through an unknown mechanism [76]. In humans, Ago proteins do not appear to selectively bind miRNA;[75] however, this is still controversial [77]. Protein-associated miRNA is stable in the blood for several weeks at minimum suggesting that the high proportion of Ago-bound miRNA is due to a lack of degradation [72,76,78]. The expression of extracellular miRNAs complexed with Ago proteins, for the most part, reflects the miRNA expression within the cell [78].

The majority of the remaining 1-10% of circulating miRNA is encapsulated within extracellular vesicles, and a small fraction is complexed with HDL [69,79]. Extracellular vesicles are characterized as a heterogeneous population of small vesicles released into the extracellular space in response to a number of stimuli including activation and apoptosis [80,81]. Extracellular vesicles may be further subcategorized as exosomes, apoptotic bodies and microparticles, based upon size and biogenesis, however, for the purposes of this review they will be collectively termed extracellular vesicles [81].

The miRNA signature of extracellular vesicles poorly correlates with the cellular miRNA signature indicating that miRNA are not passively encapsulated but actively sorted and packaged [82,83]. Little is known regarding the mechanisms of miRNA selection and loading into extracellular vesicles. Annexin 2 and Annexin 5 knockdown results in a non-specific reduction of extracellular vesicle miRNA, however the role that they play is unknown [84]. One group demonstrated that loading of miRNA into extracellular vesicles is facilitated by heterogeneous nuclear ribonucleoprotein (hnRNP) A2B1 which binds a GAGG motif on miRNA and then the complex becomes encapsulated and the miRNA is released into the lumen of the extracellular vesicles [85].

The discovery of miRNA encapsulation and transport prompted the hypothesis that cell-free miRNA participates cell-cell communication and modulates cell physiology. There are no indications that miRNA complexed with Ago proteins is uptaken and/or physiologically functional [69]. Conversely, several lines of evidence indicate that microvesicles, such as microparticles, mediate cell-cell transfer of miRNA which in turn beneficially or negatively influences cell phenotype in a miRNA dependent manner [71,82,86,87]. These data serve as a theoretical link between circulating miRNA expression and pathophysiology. Moreover, the circulating miRNA signature has been shown to be implicated in the etiology of several pathologies, including coronary artery disease and its risk factors [31,77,87–90].

PATHOPHYSIOLOGY OF CORONARY ARTERY DISEASE

Coronary artery disease (CAD) is responsible for 1 in 6 deaths domestically and is the leading cause of death domestically and worldwide [91,92]. The clinical manifestation of CAD (i.e. myocardial infarction) is due to is atherosclerotic lesion growth leading to arterial lumen narrowing and/or thrombosis as a result of atherosclerotic plaque rupture [93]. The atherosclerotic process begins with vascular smooth muscle cell migration into, and proliferation in, the vascular intima which is composed of the endothelial cell monolayer lining the vessel lumen and the internal elastic membrane surrounding the endothelium [94]. Alteration in the composition of the extracellular matrix of the intima drives the retention of blood lipids and formation of lipid pools.[95] Lipid accumulation and/or pro-inflammatory signals from neighboring cells induces endothelial activation and the characteristic expression and release of cell adhesion molecules, pro-inflammatory chemokines, cytokines and growth factors [93,96]. Macrophages recruited by the local pro-inflammatory milieu bind cell adhesion molecules on the endothelium and transmigrate

from the vessel lumen into the subendothelial space where they uptake and oxidize lipids and release signaling molecules to recruit other immune cells [94].

If lipid and/or inflammatory cell accumulation is not halted, irreversible vascular intima remodeling and pathological vascular intima thickening will occur [97]. Local hypoxia and oxidative stress can lead to the necrosis and calcification of the plaque core and/or angiogenesis in the blood vessel network (vaso-vasorum) surrounding the plaque accentuating inflammatory cell invasion [98]. Core necrosis may be preceded or followed by the replacement of the endogenous, highly compliant, vascular intima extracellular matrix with a collagen rich inelastic fibrous matrix whereby forming a fibrous cap [94,98].

These pathophysiological changes of the vasculature promote ischemia and thrombosis which underlie the clinical outcomes and sequelea of CAD: myocardial infarction, arrhythmia, ischemic heart disease, heart failure, angina pectoralis and acute coronary syndromes [93,97,99]. Ischemia due to vessel stenosis or occlusion can cause damage and disease of the tissue distal to the area of disease [97]. Abrupt narrowing of the arterial lumen causes turbulent blood flow which, in-turn, stimulates thrombosis in the region just following the lesion [100]. Atherosclerotic plaques may rupture releasing the prothrombotic content of the plaque into the vessel causing clot formation [93]. Additionally, sections of plaques may dislodge causing vessel occlusion [93].
It is well known that CAD risk is enhanced by the presence of CAD risk factors including, but not limited to, human immunodeficiency virus(HIV)-1 infection, obesity, hypertension, and diabetes [91,101–103]. For example, adults with HIV-1 have a drastically greater risk of atherosclerosis [104], CAD [105], peripheral artery disease [106] and acute myocardial infarction [107]. The pathophysiological mechanisms underlying the contribution of CAD risk factors to CAD are not

fully understood. However, proper endothelial function is critical for cardiovascular homeostasis and endothelial dysfunction is thought to play an integral role in the development of CAD [108].

Endothelial Dysfunction and Coronary Artery Disease

Endothelial dysfunction is characterized by increased endothelial permeability to lipoproteins and lipids, higher pro-coagulant molecule production, reduced capacity to regulate thrombosis and reduced vasomotor regulation [100,109,110]. Moreover, the dysfunctional endothelial cells can adopt a pro-inflammatory phenotype producing chemotactic molecules and cell adhesion molecules which stimulate macrophage recruitment, activation and translocation [97,100]. Additionally, endothelial cell senescence, oxidative stress, and apoptosis contribute to endothelial dysfunction and atherogenesis [110]. As such, endothelial dysfunction has been implicated in the etiology of CAD and associated with the etiology of almost all other cardiovascular diseases [109,111]. Indeed, endothelial dysfunction is observed in the early stages of CAD [108] and severity is positively correlated the pathophysiological progression of CAD [112,123]. Furthermore, it has been established that the endothelium can become dysfunctional in response to chronic exposure to CAD risk factors and this, in-turn, underlies the increased cardiovascular risk [108,112]. For example, HIV-1 proteins, such as glycoprotein (gp)120 and transactivator of transcription (Tat) are released into the circulation by infected memory $CD4^+$ T cells in individuals with HIV-1 [113–115]. Exposure of the endothelium to these viral proteins causes endothelial stress, injury and dysfunction [113–117]. Specifically, gp120 is cytotoxic and activates pro-apoptotic and pro-oxidative pathways resulting in endothelial dysfunction [113,116]. Additionally, Tat has been shown to induce endothelial inflammation and apoptosis as well as increase vascular permeability [114,118]. Thus, exposure of the endothelium to gp120 and Tat cause

endothelial dysfunction and, in turn, CAD pathogenesis [119–122]. However, the effects of these viral proteins on the endothelium are diverse, complex and not fully understood.

Over the past decade, several studies have characterized the influence of endothelial dysfunction and CAD on circulating miRNA expression [124–130]. Pre-clinical and clinical studies have etiologically linked alterations of the miRNA signature within tissues and the circulation to CAD [73,82,87,89,90,131–134]. Furthermore, every CAD phenotype and CAD sequelae are associated with altered miRNA expression within cells, tissues and in the circulation and the miRNA signature of each disease phenotype is not uniform [10,14,73,89,135–137]. Interestingly, several studies have demonstrated that alteration of the circulating miRNA signature can be predictive of clinical events and may aid in the prognosis and management of disease [136–141].

The expression of circulating miRNA has been investigated in a myriad of disease and development processes and has demonstrated potential as a biomarker and therapeutic target [82,88,135,142–144]. Despite this, a uniform panel of circulating miRNAs which are influenced by CAD has yet to be established. Additionally, the influence of CAD risk factors on circulating miRNA expression has yet to be fully elucidated. This is likely due to the diversity of CAD risk factors and clinical populations,[145] the common presence of comorbidities of CAD in study populations and substantial methodological differences between studies [136]. However, certain miRNAs are reproducibly observed to be dynamically regulated with endothelial dysfunction, CAD, and with its risk factors [135,146,147].

ASSOCIATIONS AND ROLES OF miRNA IN CORONARY ARTERY DISEASE

Alterations of the Circulating miRNA Signature Associated With Coronary Artery Disease

Alteration of the circulating miRNA signature has been linked with stable CAD and select miRNAs may serve as a biomarkers of early CAD [147–149]. A landmark cross-sectional, and highly cited, study by Fichtlscherer et al.,[130] demonstrated that stable coronary artery disease is associated with reduced circulating levels of miR-126, miR-17, miR-92a, miR-199a, miR-155 and miR-145 and increased expression of miR-133 and miR-208a when compared with healthy controls. Importantly, medication use, blood pressure and HDL levels were not similar between the groups and the control group was significantly younger. Gao and others demonstrated that circulating levels of miR-145 exhibit a strong inverse correlation with CAD severity, and may serve as a biomarker of early CAD [149]. Weber et al. also found that stable CAD is associated with reduced circulating expression of miR-145, along with miR-19a, miR-146a, miR-181d, miR-222, miR-29a, miR-378, and miR-150 [150]. Another study by Han and others demonstrated circulating levels of miR-34a, miR-21 and miR-23a are higher in a pre-clinical model of CAD and in humans with CAD when compared with healthy controls [151]. Importantly, a subset of these miRNAs linked with CAD are known to regulate endothelial function and may provide mechanistic insight into the development and progression of CAD.

Regulation of Endothelial Function by miRNA

Aberrant expression of a subset of miRNA, miR-126, miR-150, miR-34a, and miR-92a has been etiologically linked with atherosclerosis and CAD [10,89] though the regulation of cardiovascular homeostasis, endovascular health, and endothelial function [21,129,135,152]. Importantly, the circulating expression of these miRNA is altered in association with CAD and risk factors of CAD. Transfer of these miRNAs between cells *in vitro* and *in vivo* is known to influence endothelial cell function and modulate vascular physiology [20,71,139,153,154] serving as a

link between circulating miRNA expression and CAD. It is important to note that these are not the only miRNAs involved in the regulation of endothelial function, however, these specific miRNA are a predominate focus of ongoing research due to their well characterized role in the vasculature and their potential role in disease.

miR-126

miR-126 is enriched in the endothelium and negatively regulates endothelial inflammation and promotes angiogenesis, vascular repair and vascular stability [87]. Lower circulating expression of miR-126 is linked with heart failure, and ischemic heart disease, diabetes and hypertension. Delivery of miR-126 with apoptotic bodies through the circulation reduced plaque size and neointima formation in a mouse model (APOE-/-) of atherosclerosis [155]. Others demonstrated that delivery of miR-126 in apoptotic bodies enhanced the expression of a cytokine (CXCL12) which counteracts endothelial apoptosis and promotes vascular repair through endothelial progenitor cell recruitment. Interestingly expression of miR-126 is induced by pro-inflammatory transcription factors and is thought to limit inflammation in a negative feedback manner [133]. Circulating miR-126 expression is lower in individuals with coronary endothelial dysfunction when compared with healthy controls [156]. A study conducted by Jansen and colleagues demonstrated a direct relation between encapsulated miR-126 expression and the risk of major cardiovascular disease events [139]. Listner and colleges found that circulating expression of miR-126 inversely correlates with the presence of fibrotic plaques in humans [148]. In summary, lower circulating levels of miR-126 are associated with the development and prognosis of CVD. Furthermore, miR-126 demonstrates several anti-atherogenic functions and is pivotal to endothelial cell function.

miR-150

Atherogenesis can be initiated by unrepaired vascular damage [95]. Vascular repair is mediated by migratory endothelial progenitor cells which hone to sites of vascular injury, release angiogenic growth factors, and promote reendothelization [157]. Endothelial apoptosis and functional impairment of endothelial progenitor cells is thought to contribute to the acceleration of atherosclerosis [93,157,158]. Tang and colleges demonstrated that miR-150 is a critical modulator of apoptosis post myocardial infarction through the down-regulation of proapoptotic genes bax, egr2 and p2x7r *in vitro* and *in vivo* [159]. A elegant study conducted by Zhang et al., [71] established that miR-150 transferred between monocytes and endothelial cell enhances endothelial migration and vascular repair. The circulating expression of miR-150 is lower in individuals with CAD, acute myocardial infarction, heart failure, dilated cardiomyopathy, and ischemic cardiomyopathy when compared with healthy controls [88,136,159]. miR-150 blunts endothelial cell apoptosis and miR-150 transferred cell to cell though the circulation promotes vascular repair and CAD is associated with lower levels of circulating miR-150.

miR-34a

Endothelial senescence is recognized to promote endothelial dysfunction, atherosclerosis and CAD [160]. miR-34a positively regulates senescence through downregulation of the sirtuin system gene, SIRT1, and is known to reduce vascular repair[161], drive cardiac aging[20] and promote CAD [20,21,160]. Several studies have demonstrated miR-34a is potent promotor of cellular senescence [20,21,160]. Over expression of miR-34a impairs endothelial progenitor cell mediated vascular repair through SIRT1 inhibition *in vivo* [161]. The anti-apoptotic protein PNUTS is also

reduced by miR-34a which promotes cardiomyocyte and endothelial apoptosis [20]. The expression of miR-34a is higher in both the circulation of mice and humans with CAD when compared with healthy controls.[151] Raitoharju and colleagues isolated RNA from human vascular tissue and determined the expression of miR-34a is higher in atherosclerotic plaques compared to healthy arterial tissue [162]. Moreover, circulating expression of miR-34a is increased with CAD, myocardial infarction, and heart failure [163]. Taken together, miR-34a is integrally linked with endothelial senescence, apoptosis and dysfunction. Furthermore, higher circulating miR-34a expression is associated with CAD and its risk factors.

miR-92a

Two atheroprotective transcription factors (Kruppel-like factor 2 and 4) which dampen endothelial inflammation and promote the expression of the vasculoprotective endothelial nitric oxide synthase enzyme, are directly targeted by miR-92a [10,108]. Accordingly, miRNA-92a is known to induce endothelial expression of cell adhesion molecules, reduce endothelial nitric oxide synthase expression,[164] reduce endothelial proliferation and reduce endothelial migration thereby promoting endothelial dysfunction [152]. In a mouse model of CAD, Loyer et al,. discovered blockade of miR-92a *in vivo* results in reduced endothelial inflammation, decreased plaque size, and promoted a more stable plaque phenotype [165]. Daniel and colleagues determined that inhibition of endothelial cell miR-92a accelerated endothelial recovery and blunted leucocyte recruitment and atherosclerotic lesion formation in response to vascular injury [152]. Expression of miR-92a is higher in atheroprone areas of the aorta[164] and in atherosclerotic lesions of the aorta [165]. Indeed, the circulating level of miR-92a is higher in individuals with CAD, when compared with controls, and is lowered by statin therapy[130,166]. In summary, miR-

92a promotes endothelial inflammation, atherogenesis and higher circulating expression of miR-92a is associated with CAD.

INFLUENCE OF CORONARY ARTERY DISEASE RISK FACTORS ON CIRCULATING MIRNA EXPRESSION

Diabetes

Diabetes mellitus is characterized by a dysregulation of blood glucose levels and is a major risk factor for CAD, stroke, peripheral vascular disease, heart failure and atrial fibrillation [102]. A study of more than 800 diabetic and nondiabetic individuals revealed that diabetes is associated with reduced circulating expression of miR-126, miR-20b, miR-21, miR-24, miR-15a, miR-191, miR-197, miR-223, miR-320 and miR-486 when compared with healthy controls [129]. In a separate study Kong and others reported increased circulating levels of miR-9, miR-29a, miR-30d, miR-34a, miR-124, miR-146, and miR-375 in diabetic individuals compared with healthy controls [143]. Zhang and colleagues demonstrated that low circulating miR-126 levels predicated the development of type 2 diabetes in a 2-year prospective clinical trial [167]. At least two studies demonstrate that the diabetes was associated with higher circulating levels of miR-320a, miR-142, miR-222, miR-29a, miR-27a, and miR-375 and lower levels of miR-126, miR-197, miR-20b, miR-17, and miR-625 compared with nondiabetic controls [143,167,168]. These findings indicate that diabetes is associated with dynamic regulation of the circulating expression of several miRNAs involved in the regulation of endothelial function including miR-126 and miR-34a.

Hypertension

Hypertension (blood pressure ≥140mmHg systolic or ≥90mmHg diastolic) is a risk factor of CAD and was directly responsible for 71,942 deaths in the year 2013 [102]. The circulating miRNA signature of hypertension is not well characterized. However, a handful of studies demonstrate that the circulating expression of miR-126, and miR-296 is reduced while expression of miR-1, miR-21, miR-210 and miR-34a is higher in hypertensive individuals when compared with controls [169–171]. Out of several, at least one study has determined that the circulating expression of miR-92a, miR-1, miR-122, miR-181a, miR-221 and miR-222 is higher and expression of miR-17, miR-23a, miR-26b, miR-27a, mir-145, and miR-150 is lower in hypertensive individuals compared with normotensive individuals. Blood pressure is positively regulated, in part, by vasopressin through the rennin-angiotensin-aldosterone system. Nossent and colleagues demonstrated that patients with a mutation within the vasopressin receptor gene preventing regulation by miR-526b and miR-578 showed increased blood pressure suggesting these miRNAs play a role in blood pressure regulation [170]. Together these studies link hypertension with adverse alterations in the circulating expression of several miRNAs involved in the regulation of endothelial function including miR-126, miR-150, miR-92a and miR-34a.

Obesity

It is estimated between 31.5% and 38.1% of Americans are overweight (25<BMI≤29.9) and obese (BMI>29.9) [102]. Overweight and obesity are major risk factors for the development of heart failure, stroke and CAD [102]. Overweight and obesity may underlie the development of other CVD risk factors.[91] Several studies have determined miRNAs play a causal role in the regulation of adiposeness but only six studies have examined the circulating miRNA expression in obese and non-obese adults. None of these studies controlled for comorbidities of CAD, type 2 diabetes

or medication use between the groups. When the results from these studies are combined two or more of these studies linked obesity with higher circulating levels of miR-142, miR-140 and miR-222 and lower levels of miR-223, miR-21, miR-221, miR-125b and miR-103 [143,168,172–175]. At this time the influence of obesity on circulating miRNA expression and specifically the expression of miR-126, miR-150, miR-34a and miR-92a is unknown. Further studies are warranted to evaluate the influence of obesity on the circulating miRNA signature.

SUMMARY AND FUTURE DIRECTIONS

It is clear that miRNAs play an important role in the regulation of physiological and pathological processes. Cells actively package and release miRNAs into the circulation and mounting evidence suggests that circulating miRNAs are involved in cell-to-cell communication and regulate target cell functions. Alterations of the circulating miRNA signature is also indicative of disease development. Circulating miRNA expression is influenced by CAD and its risk factors. Furthermore, distorted circulating expression of key vascular miRNAs, including miR-126, miR-150, miR-34a, and miR-92a, may reflect and/or adversely influence endothelial function.

Many studies examining circulating miRNA expression have significant design and/or technical limitations and few studies have examined the influence of risk factors of CAD on circulating miRNA expression in the absence of other comorbidities. Currently there are no studies demonstrating the influence of obesity, if any, on circulating miRNA expression in adults. This represents a critical lack of knowledge as a greater understanding of the factors and

mechanisms contributing to the increased vascular risk associated with obesity may enhance the identification, clinical management, and treatment of CAD. Key endothelial-related miRNA are known to be altered with CAD and yet it is unclear whether these miRNAs are altered in association with risk factors of CAD. Examining the influence of risk factors of CAD on the circulating expression of key vascular miRNAs may provide further insight into the link between risk factors of CAD and endothelial dysfunction and CAD.

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CHAPTER 2

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 hours 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 and R5 gp120 and Tat significantly increased (~100%) cellular senescence vs control. X4 gp120 significantly increased cell expression of 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 (1.23±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) [1-3]. A major factor underlying the increased CVD burden with HIV-1-infection is endothelial damage and dysfunction [2,4]. 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 [3,5,6]. For example, HIV-1 gp120 induces endothelial cell apoptosis and impairs endothelial vasodilatory capacity [7-9]; and, HIV-1 Tat promotes endothelial cell activation and enhances the development of atherosclerotic lesions [10,11].

Endothelial cellular senescence is both a cause and consequence of endothelial dysfunction and atherosclerosis [12-14]. Senescent cells cease to perform functions necessary to maintain vascular homeostasis [14]. Concomitant with functional arrest, senescent endothelial cells develop a pro-inflammatory senescence-associated secretory phenotype resulting in the production and release of several cytokines and pro-inflammatory signaling molecules, such as IL-6 and TNF-α [15,16]. 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 [16]. Thus, endothelial cell senescence is regarded as a critical factor in the pathogenesis and progression of atherosclerotic vascular disease [12-14]. 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 are 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 [17]. 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 post-transcriptionally by either mRNA degradation and/or by suppressing translation [18]. It is now recognized that miRs, specifically miR-34a, miR-146a and miR-217, play a pivotal role in regulating endothelial cell senescence [19-22]. Altered expression of these senescence-associated miRs (SA-miRs) has been shown to mediate endothelial senescence under various physiologic and pathologic conditions [23]. The effect of HIV-1 viral proteins on the cellular expression of SA-miRs, however, is currently unknown.

Accordingly, the aim of the present 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) are adversely affected by these HIV-1 viral proteins.

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, USA). To reconstitute Tat, 100 mL of PBS was bubbled with compressed N_2 for 20 minutes 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, USA) and cultured in endothelial growth media (EBM-2 BulletKit)(Lonza, Basel, Switzerland) supplemented with the 100U/mL penicillin and 100µg/mL streptomycin under standard cell culture conditions (37°C and 5% CO_2). Growth media was replaced 24 hours after initial culture and every two 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, USA) and treated with media alone or media containing HIV-1 X4 gp120 (100ng/mL), R5 gp120 (100ng/mL) or Tat (500ng/mL) for 24 hours. After 24 hours cells were stained with a senescence-associated β-galactacidase cytochemical stain or harvested for RNA isolation. Viral protein concentrations were similar to circulating levels in untreated HIV-1-seropositive adults [24].

Senescence-Associated β-galactacidase Assay

Cellular senescence was quantified using cytochemical senescence-associated β-galactacidase (SA-β-gal) staining [25,26]. Briefly, subconfluent cells were washed twice with 2mL of PBS followed by a 5-minute incubation in 2mL of 2% formaldehyde and 0.2% glutaraldehyde to fix cells. Fixed cells were washed twice with 2mL of PBS and then incubated for 14 hours with 2 mL of freshly prepared staining solution (1 mg/mL 5-bromo-4-chloro-3-indolyl-*β*D-galactopyranoside in dimethylformamide, 40 mM citric acid/sodium phosphate, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 150 mM NaCl, 2 mM $MgCl_2$)(ThermoFisher, Waltham MA, USA). The staining solution was then removed and cells were washed twice with

2mL of PBS and once with 1mL of methanol and allowed to air dry. Cells were visualized by light microscopy (Zeiss, Thornwood, NY, USA) and quantified in 5 random image fields for each condition. Cells with blue cytoplasmic staining were identified as senescent positive cells. Senescent cells (%) was determined as SA-β-gal positive cells divided by the total number of cells counted [26].

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

RNA was isolated from cells ($1.0x10^5$) 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, USA) [27].

Immediately after RNA isolation, 150ng of RNA was reverse transcribed using the miScript II Reverse Transcription Kit (Qiagen, Hilden, German). RT-PCR was performed using the BioRad CFX96 Touch Real Time System along with the miScript SYBR green PCR kit (Qiagen, Hilden, Germany) and specific primers for miR-34a, miR-146a, miR-217 (Qiagen, Hilden, Germany) [28-30]. All samples were assayed in duplicate. miRNA expression was quantified using the comparative Ct method and normalized to U6 [27]. The fold change of each transcript was calculated as the $2^{-\Delta\Delta Ct}$ where fold change (AU) $=2^{-((Ct[\text{miR experimental}]-Ct[\text{RNU6experimental}]-Ct[\text{miR contol}]-Ct[\text{RNU6control}])}$.

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 4 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 amongst the HIV-1 viral proteins. Senescence in cells treated with denatured (boiled and sonicated) viral proteins was not different from control (data not shown).

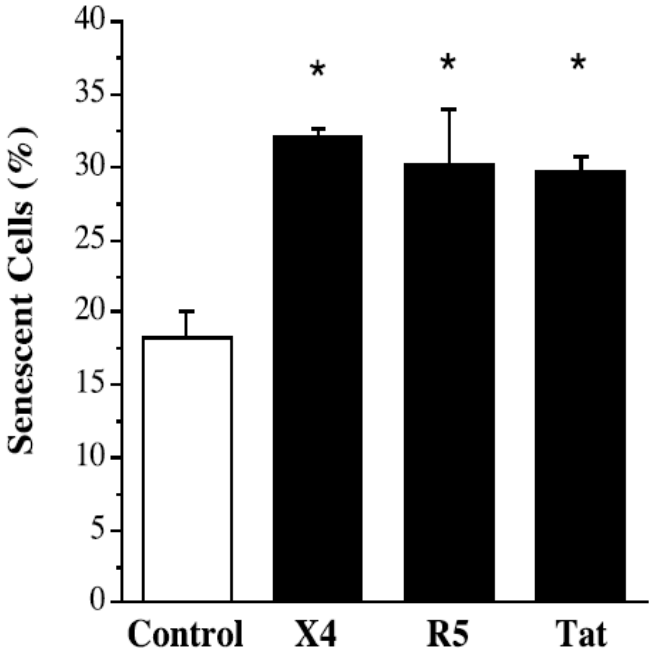

Figure 1. Endothelial cell senescence (%) following incubation with HIV-1 X4 and R5 and Tat. Values are mean±SEM (N=4). **P<0.05 vs. control*

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) (Figure 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) (Figure 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) (Figure 4).

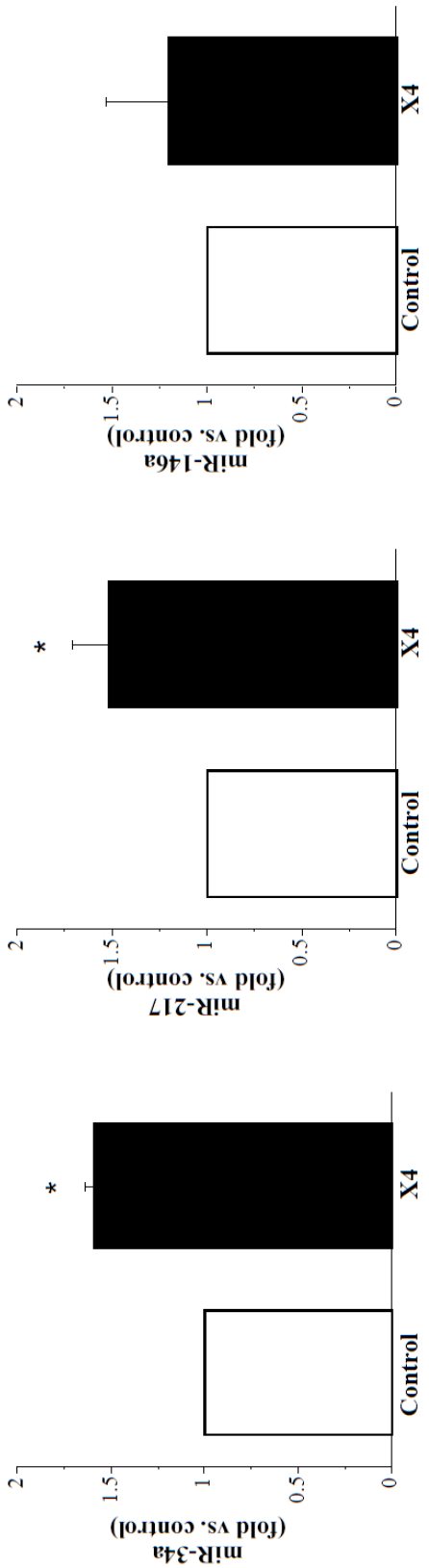

Figure 2. Expression of miR-34a, miR-217 and miR-146a in endothelial cells treated with X4 gp120 relative to control (untreated cells). Values are mean±SEM (N=4). *$P<0.05$ *vs. control*

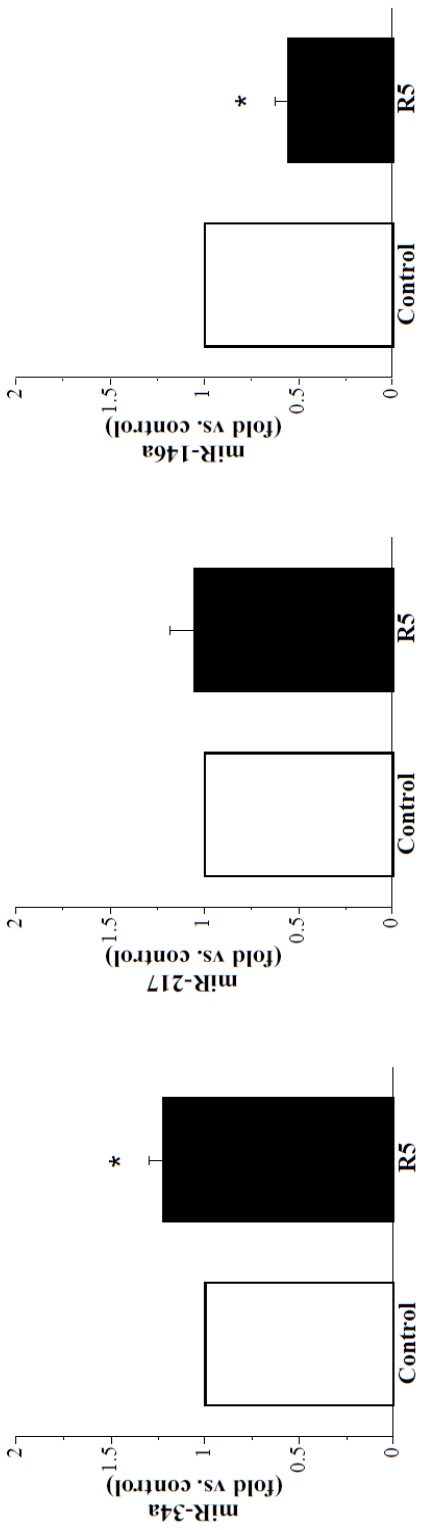

Figure 3. Expression of miR-34a, miR-217 and miR-146a in endothelial cells treated with R5 gp120 relative to control (untreated cells). Values are mean±SEM (N=4). *$P<0.05$ *vs. control*

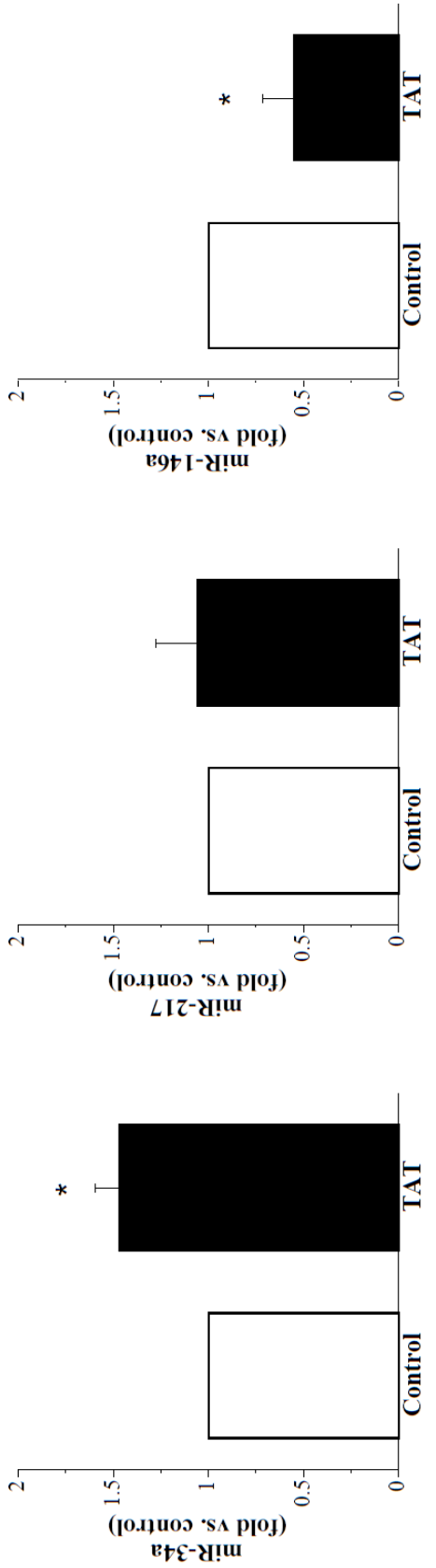

Figure 4. Expression of miR-34a, miR-217 and miR-146a in endothelial cells treated with HIV-1 Tat relative to control (untreated cells). Values are mean±SEM (N=4). *$P<0.05$ *vs. control*

DISCUSSION

The primary new findings of the present 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 [23]. For example, in addition to the release of pro-inflammatory 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 [31-34]. Furthermore, senescence negatively affects the regenerative and angiogenic capacity of the endothelium diminishing its reparative capacity and promoting atherosclerotic lesion development [12-14,35,36]. Indeed, senescent endothelial cells have been found *in vivo* at atherosclerotic sites in both the aorta and coronary arteries [13,36]. The results of the present 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 pro-senescent 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 [37] and their associated miRNAs [38]. In fact, aberrant expression of SA-miRs is now regarded as a central feature of a senescent endothelial phenotype [23,38]. In the present 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 [19,22,39]. Both miR-34a and miR-217 promote, whereas miR-146a quells endothelial cell senescence [23,38]. miR-34a is highly expressed in endothelial cells and the degree of expression increases during cell senescence [19,23,40]. miR-34a targets and down regulates sirtuin-1 (SIRT1), a major regulator of endothelial cell longevity and metabolic function [19,41,42]. SIRT1 is a class III histone deacetylase involved in the deacetylation of a variety of proteins, including NF-kB and PPAR-α [43]. SIRT1 also exerts regulatory influence on FOXO3 and p53 [19,44]. Decreased expression of SIRT1 associated with over expression of miR-34a triggers senescence in endothelial cells [19,38]. 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. [45] 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 [22]. 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 pro-senescent action of miR-34a and miR-217, miR-146a inhibits senescence by targeting proteins in the BCL-2 protein family (e.g. BCL-wL 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 [39,46]. 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 [21,46]. Thus, reduced expression of miR-146a is a hallmark feature of senescent prone endothelial cells [23,39]. In the present 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 pro-senescent cellular miR signature emerged in response to gp120 and Tat exposure that is consistent with their common senescent effects. Whilst R5 gp120 and Tat did not affect the expression of the pro-senescent miR-217, they each significantly reduced the expression of the anti-senescent miR-146a; whereas X4 gp120 increased miR-217 expression, but had no effect on miR-146a.

A limitation of the present 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 [47] and CCR5 [48], 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 [49]. Importantly, Tat is found in the peripheral blood of individuals with virus suppression on antiretroviral therapy [49], 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 [3,5,45]. 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 the present 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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CHAPTER 3

HIV-1 gp120 and TAT DERIVED MICROPARTICLES IMPAIR ENDOTHELIAL CELL FUNCTION

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ABSTRACT

The aim of this study was to determine whether: 1) human immunodeficiency virus (HIV)-1 gp120 and Tat stimulate the release of microparticles from endothelial cells; and 2) viral protein induced EMPs are deleterious to endothelial cell function: inducing endothelial cell inflammation, oxidative stress and senescence, and increasing apoptotic susceptibility. Human aortic endothelial cells (HAECs) were treated with recombinant HIV-1 proteins Bal gp120 (R5), Lav gp120 (X4) or Tat. Endothelial microparticles (EMPs) released in response to each viral protein were isolated and quantified. Fresh HAECs were treated with EMPs generated under control conditions and from each of the viral protein conditions for 24 h. EMP release was higher ($P<0.05$) in HAECs treated with R5 (141±21 MP/μL), X4 (132±20 MP/μL) and Tat (130±20 MP/μL) compared with control (61±13 MP/μL). Viral protein EMPs induced significantly higher endothelial cell release of pro-inflammatory cytokines and expression of cell adhesion molecules than control. Reactive oxygen species production was more pronounced ($P<0.05$) in the R5-, X4- and Tat-EMP treated cells. In addition, viral protein-stimulated EMPs significantly augmented endothelial cell senescence and apoptotic susceptibility. Concomitant with these functional changes, viral-protein stimulated EMPs disrupted cell expression of microRNAs: 34a, 126, 146a, 181b and 221 ($P<0.05$). These results demonstrate that HIV-1 gp120 and Tat stimulate microparticle release from endothelial cells and these microparticles confer pathologic effects on endothelial cells by inducing inflammation, oxidative stress and senescence as well as enhancing susceptibility to apoptosis. Viral protein-generated EMPs may contribute to the increased risk of vascular disease with HIV-1.

INTRODUCTION

Human immunodeficiency virus (HIV)-1 infection is associated with an increased risk and prevalence of atherosclerotic cardiovascular disease (ASCVD) [1]. The pathogenesis of HIV-1-associated atherosclerosis is complex [2,3]. Traditional risk factors and exposure to antiretroviral therapy do not fully account for the elevated risk and increased incidence and severity of atherosclerotic vascular disease associated with HIV-1 [1]. Endothelial cell activation, inflammation, dysfunction and death are primary initiating factors in the etiology of atherosclerosis [4–6] and are associated with HIV-1 infection [2,3]. Importantly, however, the direct and indirect atherogenic effects of HIV-1 on endothelial cell biology are diverse and not completely understood [2,3]. HIV-1-related proteins, such as envelope glycoprotein(gp)120 and transactivator of transcription (Tat) are thought to play a role. Released by HIV-1 infected monocytes, these proteins have been shown to disrupt endothelial cell function and jeopardize survival [3,7,8]. For example, gp120 is cytotoxic, activating pro-oxidative and proapoptotic pathways [7,9]; and, Tat has been shown to induce endothelial inflammation, monocyte adhesion and apoptosis [10,11]. Viral protein-induced microparticle release from endothelial cells may represent an additional mediator of endothelial dysfunction with HIV-1.

It is now recognized that a primary indicator of endothelial cell activation, injury and death is the vesiculation and release of endothelial microparticles [12–14]. Endothelial cell-derived microparticles (EMPs) are small (0.1-1μM in diameter) extracellular anucleod vesicles that when released can serve as both a consequence and cause of endothelial dysfunction as well as a biomarker and a predictor of vascular health and disease [12,15–17]. Clinically, elevated circulating levels of EMPs have been reported in several cardio/cerebrovascular-related diseases and risk factors stemming from, or associated with, endothelial dysfunction such as, acute coronary

syndromes [18], atherosclerosis [19], heart failure [20], stroke [21], hypertension [22], dyslipidemia [19], diabetes [23] and obesity [24]. Moreover, elevations in EMPs have been shown to be indicative of poor outcome in a variety of settings including heart transplant [20], myocardial infarction [25] and pulmonary hypertension [26]. Importantly, depending on their stimulus for release, EMPs can be pathogenic inducing and perpetuating endothelial dysfunction resulting in the development of a proatherogenic, prothrombotic vascular phenotype [14]. For example, EMPs generated under diabetic glucose conditions have been shown to impair endothelial cell nitric oxide production and ignite cellular inflammation [16,27–29]. Currently it is unknown whether HIV-1 viral proteins stimulate EMP release and, if so, whether the viral protein-derived EMPs are deleterious to endothelial cell function.

Accordingly, we tested the hypotheses that: 1) HIV-1 R5- and X4-tropic gp120 and Tat stimulate the release of microparticles from endothelial cells *in vitro*; and 2) viral protein induced EMPs are deleterious to endothelial cell function promoting endothelial cell inflammation, oxidative stress and senescence; increasing apoptotic susceptibility. Understanding the role of EMPs may provide additional mechanistic insight into the adverse vascular effects of HIV-1-associated proteins gp120 and Tat.

METHODS

Viral Proteins

Recombinant HIV-1 Tat and Bal gp120 (R5) were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAD, NIH) and HIV-1 Lav gp120 LAV (X4) was acquired from Protein Sciences Corporation (Meriden, CT, USA). To reconstitute Tat, 100mL of PBS was bubbled with compressed nitrogen gas for 20 minutes followed by the

addition of 15mg of DTT and 100mg of BSA and cooled on ice. Thereafter, Tat was dissolved in 250µL of PBS solution. The gp120s were diluted in culture media to the desired concentrations.

Cell Culture and EMP Generation

Human aortic endothelial cells (HAECs) (Life Technologies, ThermoFisher, Waltham, MA, USA) were cultured in endothelial growth media (EBM-2 BulletKit)(Lonza, Basel, Switzerland) supplemented with 100U/mL penicillin and 100µg/mL streptomycin under standard cell culture conditions (37°C and 5% CO_2). Growth media was replaced 24 h after initial culture and every two days thereafter. Cells were serially passaged after reaching 80- 90% confluence and cells were harvested for experimentation after reaching ~90% confluence on the 3rd or 4th passage. Cells were seeded into tissue culture flasks (Falcon, Corning NY, USA) and treated with media in the absence (control) and presence of HIV-1 R5 gp120 (100ng/mL), X4 gp120 (100ng/mL) or Tat (500ng/mL) for 24 hours (n=4 experimental units). Viral protein concentrations were similar to circulating levels in untreated HIV-1-seropositive adults [30]. After 24 h, media from each condition (control, R5, X4 and Tat) was collected and EMP concentration was determined by flow cytometry. Remaining media was stored at -80°C for EMP isolation and EMP-related experiments.

EMP Characterization and Enumeration

Collected media from each condition was centrifuged at 13,000xg at room temperature for 2 minutes to pellet and discard cellular debris. Thereafter, 100µL of the cell free supernatant was transferred to TruCount™ tubes (BD Biosciences, New Jersey, USA), incubated with the fluorochrome labeled antibody CD144-phycoerythrin (VEcadherin), fixed with

paraformaldehyde (ChemCruz Biochemicals, Santa Cruz, CA) and diluted with 500µL of PBS [29]. All samples were analyzed using a FACS Aria I flow cytometer (BD Biosciences). EMP size threshold was established using Megamix-Plus SSC calibrator beads (Biocytex, Marseille, France). EMPs from R5-, X4- and Tat-treated cells were determined using the formula: ([number of events in region containing EMPs/number of events in absolute count bead region] x [total number of beads per test/total volume of sample]).

EMP Treated Cells

HAECs were cultured as described above and seeded into 6-well tissue culture plates. Media containing EMPs collected from the control condition and the R5, X4 and Tat stimulated cells were centrifuged at 20,500xg for 30 minutes at 4°C to pellet EMPs [27,29]. EMPs were re-suspended in media at a concentration of $1.0x10^7$ EMP/mL. HAECs were treated with media containing EMPs from the control condition, R5, X4, or Tat treated condition for 24 hours. The concentration of EMPs used to treat the cells was equal between conditions. After treatment, cells and media were harvested for the determination of cell inflammation, oxidative stress, senescence, apoptosis and intracellular miRNA expression as well as cytokine release.

Cellular Inflammation

Concentration of tumor necrosis factor-α (TNF-α), interleukin(IL)-6 and IL-1β was quantified in the media harvested from each condition using a chemiluminescent ELISA (R&D Systems, Minneapolis, MN). Cell surface expression of intracellular adhesion molecule-1 (ICAM-1) was determined by flow cytometry. Intra-assay coefficient of variation for the chemiluminescent ELISAs was <8% for each assay.

Intracellular Oxidative Stress

HAECs were seeded in 96-well tissue culture plates (Thermo Scientific, Waltham, MA, USA) and allowed to adhere overnight. Adherent cells were washed and treated with 2',7'-dichlorofluorescin diacetate (DCFDA)(Abcam, Cambridge, MA, USA) stain (25μM) for 45 minutes. After DCFDA treatment cells were washed twice and stimulated with media or media containing X4-EMPs, R5-EMPs, or Tat-EMPs for 3 hours. Immediately thereafter, fluorescence was measured using a GEMINI EM microplate reader (Molecular Devices, Sunnyvale, CA, USA) and reported as mean fluorescence intensity (MFI)[31].

Senescence-Associated β-galactacidase Assay

Cellular senescence was quantified using cytochemical senescence-associated β-galactacidase (SA-β-gal) staining[32]. Following EMP treatment, HAECs were washed twice with PBS and incubated with 2mL of fixative (2% formaldehyde and 0.2% glutaraldehyde) for 5 minutes. Fixed cells were washed twice with PBS and then incubated for 14 hours with 2 mL of freshly prepared staining solution (1 mg/mL 5-bromo-4-chloro-3-indolyl-*β*D-galactopyranoside in dimethylformamide, 40 mM citric acid/sodium phosphate, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 150 mM NaCl, 2 mM MgCl2)(ThermoFisher, Waltham MA, USA). The staining solution was then removed and cells were washed twice with PBS and once with methanol and allowed to air dry. Cells were visualized by light microscopy (Zeiss, Thornwood, NY, USA) and quantified in 5 random image fields for each condition. Cells with blue cytoplasmic staining were identified as senescent positive cells. Senescent cells (%) was determined as SA-β-gal positive cells divided by the total number of cells counted[33].

Intracellular Active Caspase-3

After EMP treatment, harvested cells ($3.0x10^6$) for caspase-3 determination were treated with staurosporine (1µmol/L) for 3 h at 37°C and biotin-ZVKD-fmk inhibitor for 1 h at 37°C. Intracellular concentration of active caspase-3 was determined by enzyme immunoassay[34].

Intracellular miRNA Expression

Intracellular miRNA expression was determined by RT-PCR. After EMP treatment, $1.0x10^5$ cells were harvested and total cellular RNA was isolated using the miRVANA RNA isolation kit (Exiqon, Vedbake, Denmark) [35]. RNA concentration was determined using a Nanodrop Lite spectrophotometer (ThermoFisher, Waltham, MA, USA). Thereafter, 150ng of RNA was reverse transcribed using the miScript II Reverse Transcription Kit (Qiagen, Hilden, German) [35]. RT-PCR was performed using the BioRad CFX96 RT-PCR platform with the miScript SYBR green PCR kit (Qiagen, Hilden, Germany) and specific primers miR-34a, miR-126, miR-146a, miR-181b, miR-221, miR-Let-7a and U6 (Qiagen, Hilden, Germany)[35]. All samples were assayed in duplicate. miRNA expression was quantified using the comparative Ct method and normalized to U6. The fold change of each transcript was calculated as the $2^{-\Delta\Delta Ct}$ where fold change (AU) $=2^{-((Ct[miR\ experimental]-Ct[RNU6experimental]-\ Ct[miR\ contol]-Ct[RNU6control])}$.

Statistical Analysis

Differences between treatments were determined by analysis of variance (ANOVA). Where indicated by a significant *F* value, Student-Newman-Keuls post hoc tests were performed. Data are reported as mean±SEM for 4 independent HAEC experiments. Statistical significance was set *a priori* at $P<0.05$.

RESULTS

EMP release

EMP release in response to each viral protein in shown in Figure 1. The number of EMPs generated from HAECs treated with R5 (141±21 MP/μL), X4 (132±20 MP/μL) and Tat (130±20 MP/μL) was significantly higher (~120%, ~130% and ~120%; respectively) compared with EMPs release under control culture conditions (control: 61±13 MP/μL). There were no significant differences in EMP generation amongst the viral proteins.

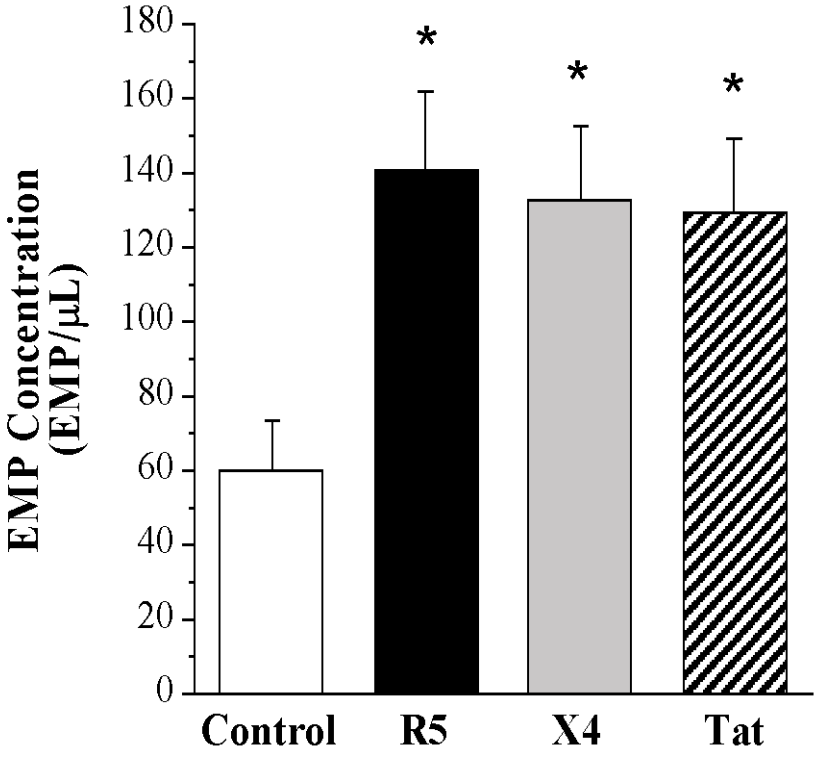

Figure 1. Effect of HIV-1 R5 gp120, X4 gp120 and Tat on endothelial cell microparticle release. Values are mean±SEM (N=4). *P<0.05 vs control (untreated) culture conditions

Endothelial Inflammation

EMPs from the viral proteins induced higher endothelial cell release of pro-inflammatory cytokines and greater upregulation in the surface expression of ICAM-1 than control (Figure 2). TNF-α release was significantly higher (~25-30%) in response to R5-EMPs (5.75±0.14 pg/mL), X4-EMPs (5.91±0.09 pg/mL) and Tat-EMPs (5.79±0.42 pg/mL) compared with control EMPs (3.94±0.18 pg/mL). Similarly, release of IL-6 and IL-1β was significantly greater (~80% and ~25%, respectively) from cells treated with R5-EMPs (103.66±4.15 pg/mL; 2.56±0.12 pg/mL, respectively), X4-EMPs (94.11±6.60 pg/mL; 2.72±0.18 pg/mL), and Tat-EMPs (94.55±11.34 pg/mL; 2.59±0.06 pg/mL) than cells treated with control EMPs (56.26±13.53 pg/mL; 2.11 ±0.14 pg/mL). Cell surface expression of ICAM-1 was approximately two-fold higher ($P<0.05$) in HAECs treated with R5-EMPs (2808.0±413.1 MFI), X4-EMPs (3016.0±343.1 MFI) and Tat-EMPs (2868.8±479.1 MFI) compared with control EMPs (1512.1±135.2 MFI). Of note, the magnitude of increase in TNF-α, IL-6 and IL-1β release and ICAM-1 expression was not significantly different between the viral protein EMP treatment conditions.

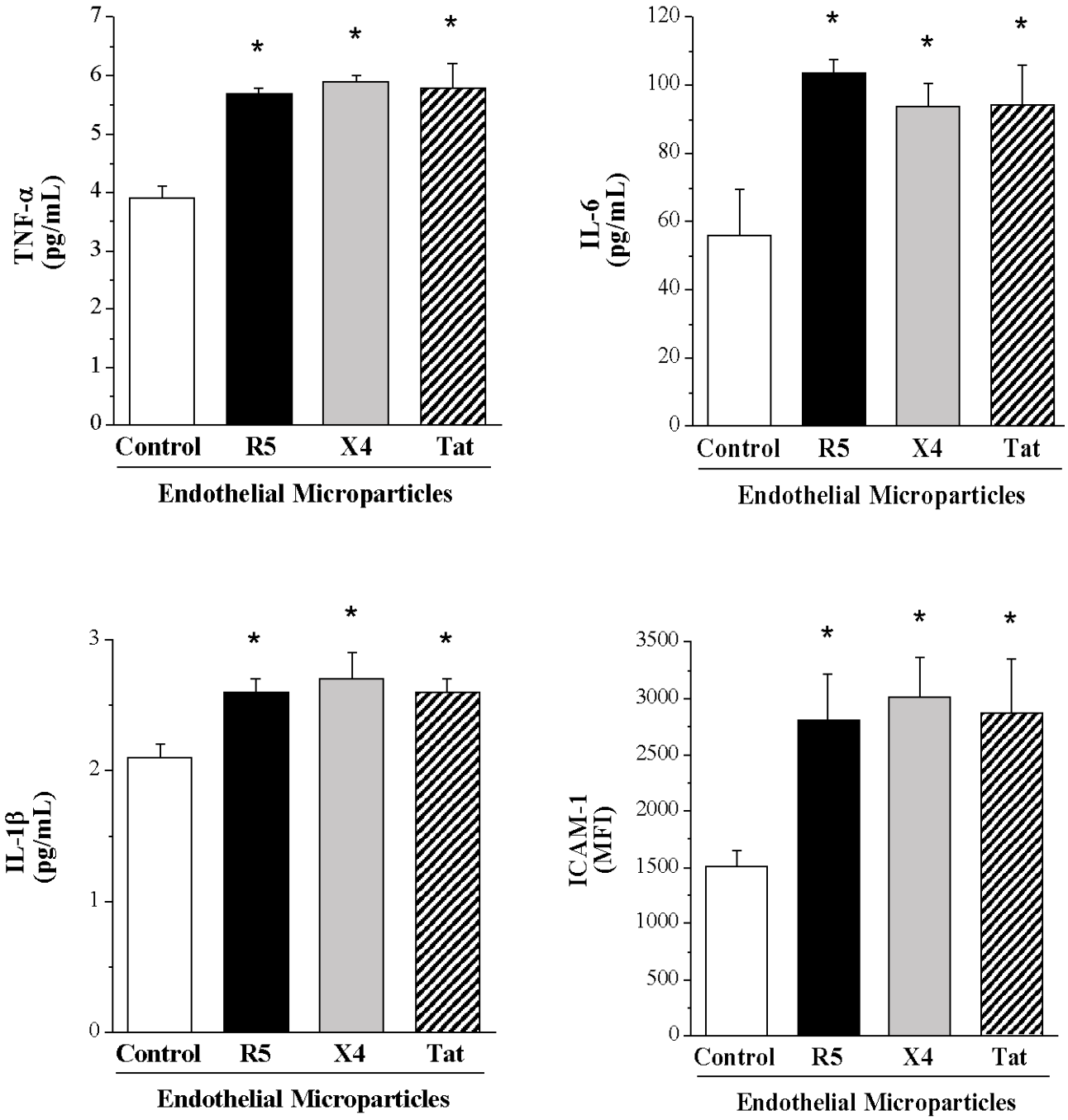

Figure 2. Effect of EMPs induced by HIV-1 R5 gp120, X4 gp120 and Tat on endothelial cell release of TNF-α, IL-6, and IL-1β as well as cell surface expression of ICAM-1. Values are mean±SEM (N=4). *P<0.05 vs EMPs from control culture condition.

Endothelial Oxidative Stress

Intracellular reactive oxygen species production was higher (~45%; P<0.05) in the R5-EMP (76.7±3.8 MFI), X4-EMP (82.8±4.2 MFI) and Tat-EMP (72.9±1.4 MFI) compared with control EMP (54.2±3.4 MFI) treated cells (Figure 3). The production of reactive oxygen species was similar between endothelial cells treated with R5-EMPs, X4-EMPs and Tat-EMPs.

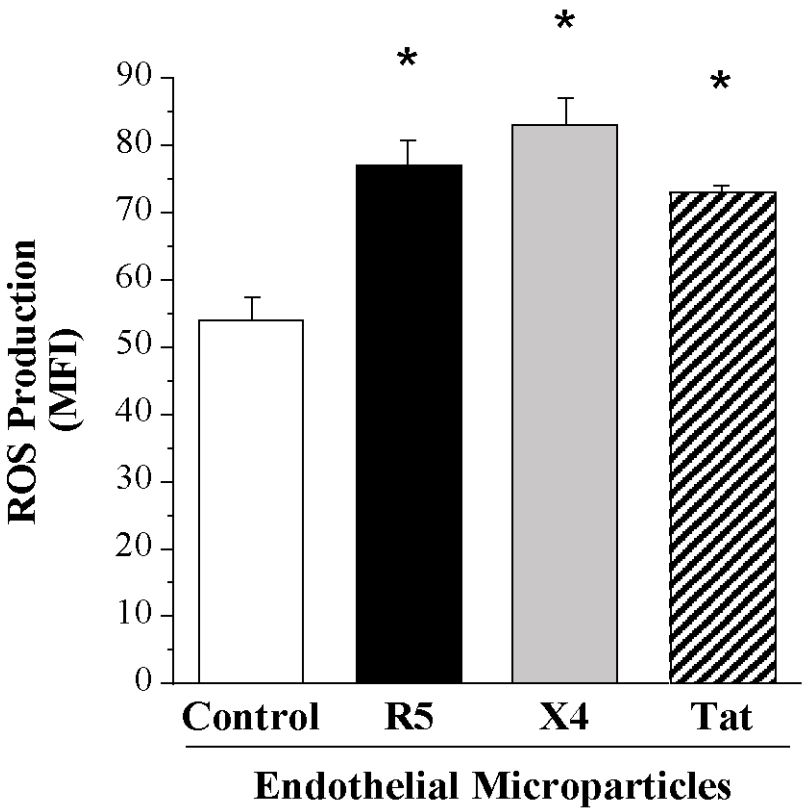

Figure 3. Effect of EMPs induced by HIV-1 R5 gp120, X4 gp120 and Tat on endothelial cell reactive oxygen species production. Values are mean±SEM (N=4). *P<0.05 vs EMPs from control culture conditions.

Endothelial Senescence

The percentage of SA-β-gal stained cells was markedly higher (~50-70%; P<0.05) in cells treated with R5-EMPs (36.1±2.2 %), X4-EMPs (32.8±2.9 %) and Tat-EMPs (31.9±2.4%) compared with control EMPs (22.3±2.4 %) (Figure 4). Endothelial cell senescence was not significantly different between the cells treated with the viral protein induced EMPs.

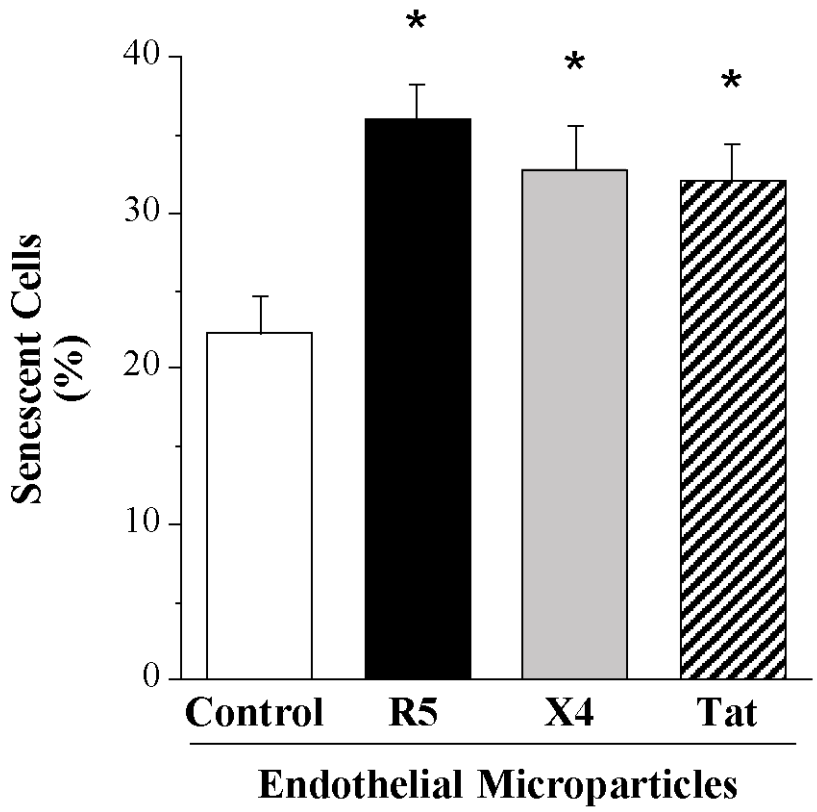

Figure 4. Effect of EMPs induced by HIV-1 R5 gp120, X4 gp120 and Tat on endothelial cell senescence. Values are mean±SEM (N=4). *P<0.05 vs EMPs from control culture condition.

Endothelial Apoptosis

Staurosporine stimulated intracellular active caspase-3 levels were significantly higher (~70%) in R5-EMP (1.61±0.15 pg/mL), X4-EMP (1.53±0.21 pg/mL) and Tat-EMP (1.59±0.22 pg/mL) treated cells compared with control EMP (0.93±0.24 pg/mL) treated cells (Figure 5). There was no significant difference in intracellular active caspase-3 concentrations between the viral protein EMP treated HAECs.

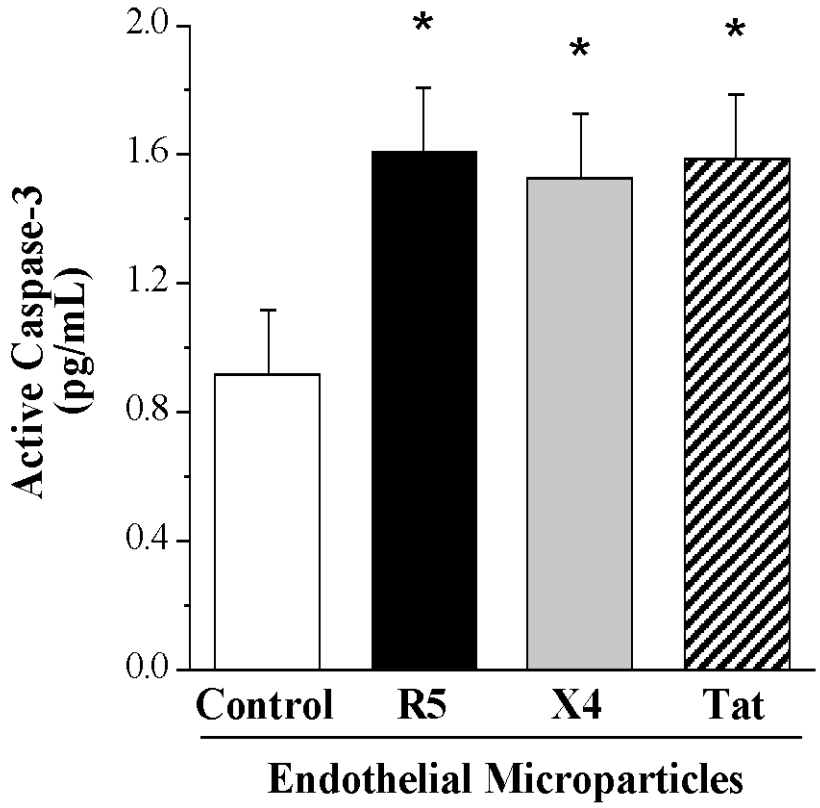

Figure 5. Effect of EMPs induced by HIV-1 R5 gp120, X4 gp120 and Tat on endothelial cell staurosporine-stimulated active caspase-3. Values are mean±SEM (N=4). *P<0.05 vs EMPs from control culture condition.

Endothelial miRNA Expression

The table presents endothelial cell expression of miR-34a, miR-126, miR-146a, miR-181b, miR-221 and miR-Let-7a in the R5-EMP, X4-EMP, and Tat-EMP treated cells. Endothelial expression of miR-34a was significantly higher (~50%) in cells treated with R5-, X4-, and Tat-EMPs compared with control EMPs. Cellular expression of miR-126, miR-146a, miR-221 and miR-Let-7a was significantly reduced (~35-120%) in response to R5-EMP, X4-EMP, and Tat-EMP. miR-181b was lower ($P<0.05$) in R5-EMP and Tat-EMP treated cells; however, there was no significant effect of X4-EMP on the cellular expression of miR-181b.

Table 1. Endothelial cell miR Expression

miRNA	R5-EMPs (fold vs control)	X4-EMPs (fold vs control)	Tat-EMPs (fold vs control)
34a	1.55±0.15*	1.48±0.12*	1.52±0.08*
126	-1.44±0.06*	-1.79±0.08*	-1.39±0.10*
146a	-2.17±0.10*	-1.52±0.10*	-1.64±0.09*
181b	-2.07±0.15*	1.19±0.06	-2.05±0.14*
221	-1.97±0.04*	-1.31±0.05*	-1.67±0.12*
Let-7a	-2.46±0.11*	-2.19±0.14*	-1.97±0.10*

Values are mean±SEM. * $p<0.05$ vs control EMP treated cells.

DISCUSSION

The primary new findings of the present study are as follows: 1) HIV-1 R5 and X4 gp120 and Tat induce microparticle release from endothelial cells; and 2) R5-, X4- and Tat-induced EMPs have deleterious effects on endothelial cell function. Indeed, viral protein-derived EMPs promote a proinflammatory, pro-oxidative, prosenescent and proapoptotic endothelial phenotype. To our knowledge, this is the first study to determine the effects of HIV-1-associated proteins on

microparticle release from endothelial cells and the impact of viral protein-induced EMPs on endothelial cell function. EMPs released in response to gp120 and Tat provide novel insight into potential mechanisms contributing to the increased risk and prevalence of endothelial dysfunction and, in turn, atherosclerotic vascular disease with HIV-1 [3].

Indication that EMPs may be causative agents in vascular disease initiation and progression stem from their numerical increase in almost all vascular-related clinical and sub-clinical conditions as well as their functional properties under pathologic conditions [12,15]. The release of EMPs is a result of plasma membrane budding due to disruption of membrane phospholipid asymmetry, cytoskeleton protein destabilization and loss of functional integrity [13]. Under normal healthy conditions endothelial cells will release low levels of microparticles to help maintain cell homeostasis and aid in cell-to-cell communication [36–38]. However, under conditions of endothelial activation, injury or apoptosis EMP vesiculation and release is upregulated and in most cases the functional phenotype of the EMP is dictated by the pathologic nature of the triggering stimulus [39,40]. Clinical and experimental studies have demonstrated that under pathological conditions EMP release is increased and the released EMPs can engage in a vicious cycle amplifying and perpetuating the deleterious vascular effects of the primary stimulus [27,41,42]. In the present study, we demonstrate, for the first time, that HIV-1 R5 and X4 gp120 and Tat significantly increase EMP release from cultured endothelial cells. Interestingly the magnitude of increase in EMPs in response to each viral protein was almost identical (~135%), suggesting common release mechanisms. Indeed, both gp120 and Tat have been shown to activate various signaling proteins involved in EMP formation and release. For example, gp120 binding of CCR5 and/or CXCR4 [43] results in the activation of p38 mitogen-activated protein kinase (MAPK) and caspase-2 [7,9], key initiators of EMP formation and release

under conditions of cell activation and increased apoptotic susceptibility [40,44]. P38 MAPK activation and caspase-2 provoke cytoskeletal rearrangement, stimulate micropartcle vesiculation and initiate release [40,44]. In addition, Tat has also been shown to activate p38 MAPK and caspase-2 through either binding with cell surface receptors or interaction with cytosolic signaling proteins [3,8,10].

Endothelial activation and inflammation is a harbinger of endothelial dysfunction and pivotal to the development of atherosclerosis [6,45]. Inflammation mediators such as cell adhesion molecules and cytokines promote the recruitment, attachment and translocation of inflammatory cells into the sub-endothelial space and the subsequent retention of lipids thereby initiating atherosclerotic lesion development [46]. Upregulation of cell adhesion molecules on the surface of the endothelium is an early and sensitive indicator of endothelial cell activation, distress and inflammation [45]. Herein, we demonstrate that R5-, X4- and Tat-induced EMPs significantly increased the surface expression of ICAM-1 (~30%) on treated HAECs. Increased expression of ICAM-1 on the surface of endothelial cells is known to facilitate greater luminal leukocyte interaction, adhesion and migration into the subendothelial space, increasing the propensity for atherosclerosis [45]. In addition to increasing ICAM-1 expression on the endothelial cell surface, each of the viral protein-derived EMPs also induced significant endothelial release of proinflammatory cytokines. Concentrations of TNF-α (~25%), IL-6 (~80%) and IL-1β (~25%) were markedly higher in the media of cells treated with R5-, X4- and Tat-EMPs. Increased production and release of TNF-α, IL-6 and IL-1β by the endothelium has been linked with the initiation and development of endothelial dysfunction and, ultimately, atherosclerotic lesion formation [6,45]. Interestingly, the observed inflammatory effects of viral protein induced EMPs on endothelial cells, is similar to the endothelial effects of the viral proteins themselves and may

contribute to the reported elevations in circulating levels of TNF-α, IL-6, IL-1β and soluble ICAM-1 in HIV-1-seropositive adults [47,48].

Increased oxidative stress often accompanies a hyper-inflammatory state [49]. As such, it is not surprising that endothelial ROS production was markedly increased (~45%) in cells treated with either R5-, X4- or Tat-EMPs. Excess ROS production can damage or modify intracellular proteins and react with nitric oxide to form peroxynitrite ($^{-}$ONOO) [50] impairing cell function and viability [49,50]. In fact, oxidative stress has been implicated as a key mechanism underlying viral protein-mediated endothelial dysfunction [9,51]. This deleterious effect of gp120 and Tat on the endothelium may also be instigated by the microparticles they generate.

Increased cellular senescence and apoptosis susceptibility often mark the penultimate stage in declining cell vitality, function and survival [5,52]. The initiation of senescent and/or apoptotic processes and pathways can lead to pathologic circumstances depending on the stimulus. In the case of endothelial cells, senescence and apoptosis result in the arrest and/or termination of key atheroprotective functions rendering the endothelium prone to atherosclerosis and thrombosis [5,52]. Both human and animal studies have demonstrated that endothelial cell senescence is remarkably higher at sites of coronary and aortic atherosclerosis compared with non-diseased areas of the vessel [4,53]. Indeed, a high preponderance of senescent endothelial cells have been reported *in vivo* at atherosclerotic sites in both the aorta and coronary arteries [4,53]. We [34,35] and others [54,55] have previously demonstrated that gp120 and Tat induce both a prosenescent and proapoptotic endothelial phenotype. The results of the present study compliment and significantly extend these findings by demonstrating that EMPs generated by each of these viral proteins also provoke endothelial cell senescence and heightened apoptotic susceptibility. The percentage of SA-β-gal stained HAECs was substantially higher in the cells exposed to R5-, X4-

and Tat-induced EMPs. Concomitant with the increase in cell senescence, the viral protein-related EMPs augmented endothelial cell apoptotic susceptibility. In response to the apoptotic stimulus, staurosporine, intracellular active caspase-3 concentrations were significantly higher in the cells incubated with each of viral protein stimulated EMPs compared with control condition EMPs. Concentrations of active caspase-3, the so called "executioner molecule" in the hierarchy of caspases, provides specific and sensitive indication of the apoptotic tendency of a cell [56]. Interestingly, the degree of cell senescence and active caspase 3 noted in the present study is comparable to the levels we have previously reported in response to R5, X4 and Tat [35,54]; providing further evidence that EMPs generated by these viral proteins are as toxic to the endothelium as the direct endothelial effects of the proteins per se.

The mechanisms underlying the profound deleterious effects of the viral protein-derived EMPs on endothelial cell function are unknown. To identify potential mechanisms and possible pathways underlying the proinflammatory, pro-oxidative, prosenescent and proapoptotic effect of R5-EMPs, X4-EMPs and Tat-EMPs we examined changes in endothelial cell microRNA (miR) profiles. miRs play a central role in regulating vascular health and function through the post-transcriptional regulation of gene expression [57]. Altered endothelial cell expression of vascular- and inflammation related miRs, specifically miR-34a, miR-126, miR-146a, miR-181b, miR-221 and miR-Let-7a, has been shown to contribute mechanistically to endothelial inflammation, oxidative stress, senescence and apoptosis [57–59]. In regard to viral protein EMP-mediated changes in cell inflammation, the expression of miR-146a and miR-221 was markedly disrupted by each of the viral protein EMPs. Both miR-146a and miR-221 expression was significantly lower in the R5-, X4- and Tat-EMP treated cells. The reduction in miR-146a across all the viral protein EMP conditions is particularly striking because miR-146a directly targets

activators of nuclear factor-κB (NF-κB), such as TRAF-6 and IRAK-1[60,61], thereby suppressing its activation. NF-κB is a primary pro-inflammatory transcription factor regulating cytokines such as TNF-α IL-6 and IL-1β as well as the cellular expression of ICAM-1 [60,61]. Thus, reduction in miR-146a expression may be an underlying factor contributing to the observed increase in soluble cytokine release and ICAM-1 expression observed in the R5-, X4- and Tat-EMP treated cells. Interestingly, miR-181b expression was diminished (~100%) in response to EMPs induced by R5 gp120 and Tat, but not X4 gp120. miR-181b directly targets the NF-κB transporter importin-α3, suppressing NF-κB translocation from the cytoplasm to the nucleus and, in turn, NF-κB-mediated transcriptional activity [57]. Reasons for the disparate effect of X4-EMPs on cellular miR-181b expression compared with R5- and Tat-EMPs are not readily apparent. However, disparities in the vascular effects of R5 and X4 are not uncommon and have been noted in other experimental conditions [43]. In addition to reductions in the aforementioned miRs, expression of miR-34a was higher, whereas miR-126 and miR-Let7a expression was lower in the cells treated with each of the viral protein-derived EMPs. Elevation in miR-34a expression promotes cellular senescence and apoptosis through the negative regulation of sirtuin-1 (SIRT-1) and the anti-apoptotic protein BCL-2, respectively [59]. Lower miR-126 and miR-Let7a expression further fosters a proapoptotic cellular phenotype through dysregulation of caspase-3 activity [62,63]. Clearly, future studies are required to more carefully dissect the mechanisms responsible for the deleterious effects of gp120 and Tat induced EMPs. The observed disruption in miR expression provides valuable clues and potential cell signaling pathways and proteins to target.

In conclusion, the results of this study demonstrate that HIV-1 gp120 and Tat protein induce microparticle release from endothelial cells and these microparticles confer pathologic

effects on endothelial cells by inducing inflammation, oxidative stress and senescence as well as enhancing susceptibility to apoptosis. It is well established that these phenotypic changes in endothelial cells are associated with, and mediate, the development of atherosclerosis and thrombosis [2,4,40]. Although we did not determine the combined effects of these viral proteins on EMP release and function; the noted independent effects of HIV-1 R5 and X4 gp120 as well as Tat on EMPs provides novel additional insight regarding the atherogenic properties of these endothelial toxic viral proteins.

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COMPETING INTERESTS

The authors have no conflicts of interest to disclose.

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CHAPTER 4

CIRCULATING MICROPARTICLES ARE ELEVATED WITH HIV-1 AND ARE DELETERIOUS TO ENDOTHELIAL CELL FUNCTION

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ABSTRACT

Background: Circulating cell-derived microparticles have emerged as biomarkers and effectors of vascular health and disease. Shed in response to various stimuli, microparticles have been shown to directly impact endothelial cell function. The aims of the present study were to determine: 1) if circulating microparticles are elevated in antiretroviral (ART)-treated HIV-1-seropositive adults; and 2) the effects of microparticles isolated from ART-treated HIV-1-seropositive adults on endothelial cell function, in vitro.

Methods: Circulating levels of endothelial (EMP)-, platelet (PMP)-, monocyte (MMP)- and leukocyte (LMP)-derived microparticles were determined by flow cytometry in plasma samples from 15 healthy and 15 antiretroviral therapy (ART)-treated HIV-1-seropositive men. Human umbilical vein endothelial cells (HUVECs) were cultured and treated with microparticles from individual subjects for 24 hr to determine their effects on endothelial cell inflammation, oxidative stress, senescence and apoptosis.

Results: Circulating concentrations of EMPs, PMPs, MMPs and LMPs were significantly higher (~50-140%) in the HIV-1-seropositive compared with healthy men. Microparticles from HIV-1-seropositive men induced significantly greater IL-6 and IL-8 (~20% and ~35%, respectively) release and NF-κB expression compared with micorparticles from healthy men. Expression of anti-inflammatory miR-146a and miR-181b was suppressed by HIV-1-seropositive microparticles. Intracellular reactive oxygen species production (ROS) and expression of ROS-related chaperone protein Hsp70 were both markedly elevated in cells treated with microparticles from the HIV-1-seropositive men. In addition, microparticles from HIV-1-seropositive men induced a prosenescent and proapoptotic endothelial phenotype. The percentage of senescent cells was significantly higher and SIRT1 expression lower in cells treated with HIV-1-related

microparticles. Whereas, cellular levels of caspase-3 were significantly higher in cells treated with microparticles from HIV-1-seropositive men compared with those treated with microparticles from the healthy men.

Conclusion: Circulating concentrations of EMPs, PMPs, MMPs and LMPs are elevated in ART-treated HIV-1-seropositive men. In addition, microparticles from ART-treated HIV-1-seropositive men adversely affect endothelial cells promoting cellular inflammation, oxidative stress, senescence and apoptosis. Circulating microparticles may contribute mechanistically to the increased risk and accelerated rate of atherosclerotic vascular disease associated with HIV-1.

INTRODUCTION

Since the introduction of effective antiretroviral therapy (ART) and consequent improvement in life expectancy, cardiovascular disease (CVD), in particular atherosclerotic cardiovascular disease, has emerged as a major cause of morbidity and mortality in HIV-1-infected individuals. HIV-1-seropositive adults have nearly twice the risk for atherosclerosis and myocardial infarction than uninfected adults [1,2], and the incidence of coronary heart disease, myocardial infarction and stroke are expected to continue to rise with an aging HIV-1 population [3–5]. The mechanisms underlying the increased risk of atherosclerosis in HIV-1-infected individuals are not completely understood. Traditional risk factors and exposure to ART do not fully account for the increased incidence and severity of vascular disease and events associated with HIV-1 [3]. For example, autopsy findings from HIV-1-positive children and young adults have reported clinically relevant atherosclerotic lesions and endovascular injury in peripheral, coronary and cerebral arteries, in the absence of CVD risk factors [6,7].

Endothelial cell dysfunction is a primary initiating event in the etiology of atherosclerosis [8,9] and is ubiquitous with HIV-1 infection [4,10]. Although not a target of the virus, HIV-1-infection promotes a proatherogenic endothelial phenotype characterized by increased cellular inflammation, oxidative stress and apoptosis and diminished nitric oxide production [11,12]. Factors underlying the profound endothelial cell dysfunction associated with HIV-1-infection are not well defined.

The vascular endothelium is significantly influenced by circulating cell-derived microparticles [13–16]. Microparticles are small (100 to 1000 nm) membrane vesicles that are released into the circulation by various cell types (e.g. endothelial cells, platelets, leukocytes, monocytes) in response to a myriad of physiologic and pathologic stimuli that induce cell

activation, injury and apoptosis [13,16]. The initial notion that microparticles in the circulation were merely "inert cellular debris" rapidly changed when it became apparent that circulating microparticles can trigger a proatherogenic endothelial cell phenotype by disrupting endothelial nitric oxide production, inducing endothelial inflammation, oxidative stress and senescence as well as altering apoptosis [17–20]. In addition, microparticles have been shown to contribute to plaque development, thrombogenicity and instability [21]. Importantly, the number, size, antigenic compositions and biological effects of microparticles are largely dictated by stimulus for release [16,22,23]. Under healthy conditions, cells constitutively release microparticles that aid in cell-to-cell communication, activate repair or defense mechanisms, and/or stimulate immune responses [22,23]. Under pathologic conditions, however, microparticles are generally released in greater number and their functional phenotype is more likely to evoke and perpetuate pathologic cellular response [16,24,25]. The numerical and functional phenotype of circulating microparticles in HIV-1-seropositive adults may represent important mediating factors underlying HIV-1-associated endothelial dysfunction.

Accordingly, the aims of the present study were to determine: 1) if circulating microparticles are elevated in ART-treated HIV-1-seropositive adults; and 2) the effects of microparticles isolated from ART-treated HIV-1-seropositive adults on endothelial cell function, in vitro. We hypothesized that circulating levels of endothelial (EMP)-, platelet (PMP)-, monocyte (MMP)- and leukocyte (LMP)-derived microparticles are elevated in ART-treated HIV-1-seropositive adults; and that microparticles from ART-treated older HIV-1-seropositive adults will induce greater endothelial cell activation, inflammation, oxidative stress, senescence and apoptotic susceptibility than microparticles from healthy adults.

METHODS

Subjects

Thirty sedentary, non-obese young and middle-aged adult men (age: 24-64 years) were studied: 15 healthy and 15 HIV-1-seropositive. All HIV-1-seropositive men were receiving ART regimens in conformance with contemporary guidelines from the U.S. Department of Health and Human Services. These included integrase-inhibitor (n=9), non-nucleoside reverse transcriptase inhibitor (n=5) and protease inhibitor based regimens (n=1).

In addition, all HIV-1-seropositive men had documented virologic suppression (<50 copies HIV-1 RNA/mL) for at least 1 year. All subjects were normotensive, non-smokers and free of overt cardiovascular and metabolic disease assessed by medical history, resting and exercise ECGs and fasting blood chemistry. Prior to participation in the study, all subjects had the research study and its potential risk and benefits explained before providing written informed consent according to the guidelines of the University of Colorado Boulder. This study was approved by the University of Colorado Institutional Review Board.

Body Composition and Metabolic Measures

Body mass was measured to the nearest 0.1 kg using a medical beam balance. Percent body fat was determined by dual energy X-ray absorptiometry (Lunar Corp., Madison, WI, USA). Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Minimal waist circumference was measured according to published guidelines [26]. Fasting plasma lipid, lipoprotein, glucose, and insulin concentrations were determined using standard techniques.

Microparticle Identification and Isolation

Microparticle isolation and identification was performed as previously described [27]. Venous blood from an antecubital vein was collected into tubes containing sodium citrate and centrifuged at 1500g for 10 min at room temperature. Plasma was collected and stored at -80°C for batch analysis and microparticle isolation. For the characterization and quantification of circulating microparticle subspecies, all plasma samples were centrifuged at 13,000g for 2 min and 200 µL was transferred to a TruCount tube (BD Biosciences, Franklin Lakes, NJ, USA). Microparticle subspecies were determined using markers indicative of endothelial (EMP: $CD62E^+$), platelet (PMP: $CD62P^+$), monocyte (MMP: $CD14^+$) and leukocyte (LMP: $CD45^+$) cell lineage. Samples were incubated with fluorochrome-labeled antibodies for 20 min in the dark at room temperature. Following incubation, samples were fixed with 2% paraformaldehyde (ChemCruz Biochemicals, Santa Cruz, CA, USA) and diluted with PBS. Thereafter, all samples were analyzed using a FACS Aria I flow cytometer (BD Biosciences). Microparticle size threshold was established using Megamix-Plus SSC calibrator beads (Biocytex, Marseille, France) and only events >0.16 µm and <1 µm in size were counted. The concentration of microparticles was determined using the formula: ([number of events in region containing microparticles/number of events in absolute count bead region] x [total number of beads per test/total volume of sample]).

To isolate microparticles from each subject sample for use in cell experiments, plasma from the sodium citrate tubes was centrifuged at 13,000g for 2 min to remove cellular debris and then re-centrifuged at 20,500xg for 30 minutes at 4°C to pellet microparticles [28]. The pelleted microparticles were then re-suspended in media and the concentration of microparticles in the media was determined by FACS.

Cell Culture and Microparticle Treatment

Human umbilical vein endothelial cells (HUVECs) (Life Technologies, ThermoFisher, Waltham, MA, USA) were cultured in endothelial growth media (EBM-2 BulletKit)(Lonza, Basel, Switzerland) supplemented with the 100U/mL penicillin and 100µg/mL streptomycin under standard cell culture conditions (37°C and 5% CO2). Growth media was replaced 24 h after initial culture and every two days thereafter. Cells were serially passaged after reaching 80-90% confluence and cells were harvested for experimentation after reaching ~90% confluence on passages 3-4. For experimentation, HUVECs were seeded into 6-well tissue culture plates with media containing microparticles from either a HIV-1-seronegative or HIV-1-seropositive adult for 24 hours. After treatment, cells and media were harvested for the determination of cellular protein expression, miRNA expression and soluble cytokine release.

Intracellular Protein Expression

Whole cell lysates were obtained from microparticle treated HUVECs for the quantification of intracellular proteins. HUVECs harvested after microparticle treatment were washed in ice cold PBS and incubated in ice-cold RIPA buffer containing protease and phosphatase inhibitors (ThermoFisher) for 15 minutes [29]. Cell lysates were sonicated for 20 seconds (four 5 second cycles spaced by 90 seconds between each cycle) and incubated on ice for an additional 15 min [29]. Thereafter, cell lysates were centrifuged at 13,000g at 4°C for 7 min and the supernatant were collected. Protein concentration was determined using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Protein expression was measured by capillary electrophoresis immunoassay (Wes, ProteinSimple, San Clara, CA, USA). Briefly, 2-3ng of cell

lysate was combined with a provided sample master mix (ProteinSimple, Santa Clara, CA) containing of 1x sample buffer, 1x fluorescent molecular weight markers, and 40 mM DTT. Samples were vortexed and heated at 95°C for 5 minutes prior to combining with blocking solution, primary antibodies, horseradish peroxidase-conjugated secondary antibody, chemiluminescent substrate, and separation and stacking matrices for automated electrophoresis (375V for 25 min) and immunodetection using the Wes system (ProteinSimple, Santa Clara, CA). Protein expression was quantified as peak area for the protein of interest normalized to peak area of β-actin in the sample. Rabbit primary antibodies against NFκB p65 (D14E12), phospho (p)-NFκB p65 (Ser536)(93H1), Caspase-3 (8G10) and Cleaved Caspase-3 (Asp175)(5A1E), sirtuin (SIRT)-1 (D1D7) (diluted 1:50, 1:25, 1:25, 1:25 and 1:50; respectively), (Cell Signaling Technologies, Danvers, MA, USA); eNOS (#PA1-037), phospho (p)-eNOS (Ser1177)(#PA5-35879, 1:25), β-actin (#PA1-16889) and tPA (#PA5-51927) (diluted 1:500, 1:25, 1:25, 1:300, and 1:25 respectively) (Thermo Fisher, Waltham, MA, USA) were used. Initial titrations were performed to optimize antibody and total protein concentration for each protein.

Intracellular Oxidative Stress

For the determination of intracellular oxidative stress, HUVECs were seeded in 96-well tissue culture plates (Thermo Scientific, Waltham, MA, USA) and allowed to adhere overnight. Adherent cells were washed and treated with 2',7'-dichlorofluorescin diacetate (DCFDA)(Abcam, Cambridge, MA, USA) stain (25μM) for 45 minutes. After DCFDA treatment, cells were washed twice and stimulated with media or media containing microparticles for 3 hours. Immediately thereafter, fluorescence was measured using a GEMINI

EM microplate reader (Molecular Devices, Sunnyvale, CA, USA) and reported as the percentage relative to control [30].

Senescence-Associated β-galactacidase Assay

Cellular senescence was quantified using cytochemical senescence-associated β-galactacidase (SA-β-gal) staining. Following microparticle treatment, HUVECs were washed twice with PBS and incubated with 2mL of fixative (2% formaldehyde and 0.2% glutaraldehyde) for 5 minutes. Fixed cells were washed twice with PBS and then incubated for 14 hours with 2 mL of freshly prepared staining solution (1 mg/mL 5-bromo-4-chloro-3-indolyl-*β*D-galactopyranoside in dimethylformamide, 40 mM citric acid/sodium phosphate, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 150 mM NaCl, 2 mM MgCl2)(ThermoFisher, Waltham MA, USA). The staining solution was then removed and cells were washed twice with PBS and once with methanol and allowed to air dry. Cells were visualized by light microscopy (Zeiss, Thornwood, NY, USA) and quantified in 5 random image fields containing a minimum of 100 per field for each condition. Cells with blue cytoplasmic staining were identified as senescent positive cells. Senescent cells (%) was determined as SA-β-gal positive cells divided by the total number of cells counted [31].

Intracellular miRNA Expression

Intracellular miR expression for specific miRs associated with cellular inflammation (mir-126, miR-146a and miR-181b), senescence (miR-34a), apoptosis (miR-126, miR-Let-7a) and eNOS (miR-21, miR-126 and miR-155) was determined by RT-PCR [31]. After microparticle treatment, $1.0x10^5$ cells were harvested and total cellular RNA was isolated using the miRVANA

RNA isolation kit (Exiqon, Vedbake, Denmark). RNA concentration was determined using a Nanodrop Lite spectrophotometer (ThermoFisher, Waltham, MA, USA). Thereafter, 150 ng of RNA was reverse transcribed using the miScript II Reverse Transcription Kit (Qiagen, Hilden, German) [32–34]. RT-PCR was performed using the BioRad CFX96 RT-PCR platform with the miScript SYBR green PCR kit (Qiagen, Hilden, Germany) and specific primers miR-21, miR-34a, miR-92a, miR-126, miR-146a, miR-155, miR-181b, miR-Let-7a and U6 (Qiagen, Hilden, Germany) [31]. All samples were assayed in duplicate. miRNA expression was quantified using the comparative Ct method and normalized to U6 [31]. The relative expression of each transcript was calculated as the $2^{-\Delta Ct}$ where $2^{-((Ct[miR]-Ct[RNU6])}$ and presented as arbitrary units (AU)

Cytokine Release

Concentration of interleukin(IL)-6 and IL-8 was quantified in the media from microparticle treated cells using chemiluminescent ELISA (R&D Systems, Abingdon, UK)[35]. Intra-assay coefficient of variation for the chemiluminescent ELISAs was <8% for each assay**.**

Statistical Analysis

Group differences in subject characteristics, circulating microparticle concentrations, cellular protein expression, miRNA expression, oxidative stress, and senescence were determined by analysis of variance (ANOVA). Pearson correlations were determined between variables of interest. Data are reported as mean±SEM. Statistical significance was set *a priori* at $P<0.05$.

RESULTS

Subject Characteristics

Selected subject characteristics are presented in the Table. There were no significant group differences in age, anthropometric or hemodynamic variables. Indeed, body composition and blood pressure were similar between the healthy and seropositive men. Although plasma triglyceride concentrations were slightly, albeit significantly, higher in the seropositive men, there were no significant group differences in plasma cholesterol concentrations or glucose and insulin levels.

Table 1. Selected subject characteristics

Variable	Healthy (*n*=15)	HIV-1-Seropositive (*n*=15)
Age (yr)	49±3	46±2
Body Mass (kg)	78.4±2.4	81.3±2.6
BMI (kg/m^2)	25.1±0.7	26.3±0.7
Body Fat (%)	24.0±1.2	26.2±1.7
Systolic Blood Pressure, (mmHg)	118±2	122±3
Diastolic Blood Pressure (mmHg)	71±3	76±2
Total Cholesterol (mg/dL)	186.9±7.8	174.0±8.1
LDL-C (mg/dL)	121.2±5.5	104.0±8.1
HDL-C (mg/dL)	46.3±2.2	43.2±2.7
Triglycerides (mg/dL)	87.4±9.5	146.5±14.7*
Glucose (mg/dL)	88.4±1.8	89.8±2.4
Insulin (μU/ml)	6.9±0.7	7.9±1.1
Average Sleep (h/night)	7.0±0.2	7.3±0.4
$CD4^+$ Count ($cells/mm^3$)		615±82
Viral Load (copies/mL)		<50

Values expressed as Mean±SE. BMI: body mass index. LDL-C: low-density lipoprotein. HDL-C: high-density lipoprotein. *P<0.05

Circulating Microparticles

Circulating levels of EMPs, PMPs, MMPs and LMPs in the healthy and HIV-1 seropositive men are shown in Figure 1. The concentration of each microparticle subspecies was markedly higher ($P<0.05$) in the HIV-1 infected men: EMPs (~50%: 140±20 vs 94±13 MP/μL); PMPs (~140%: 131±18 vs 55±9 MP/μL); MMPs (~80%; 175±34 vs 97±20 MP/μL); and LMPs (~75%; 551±45 vs 316±48 MP/μL).

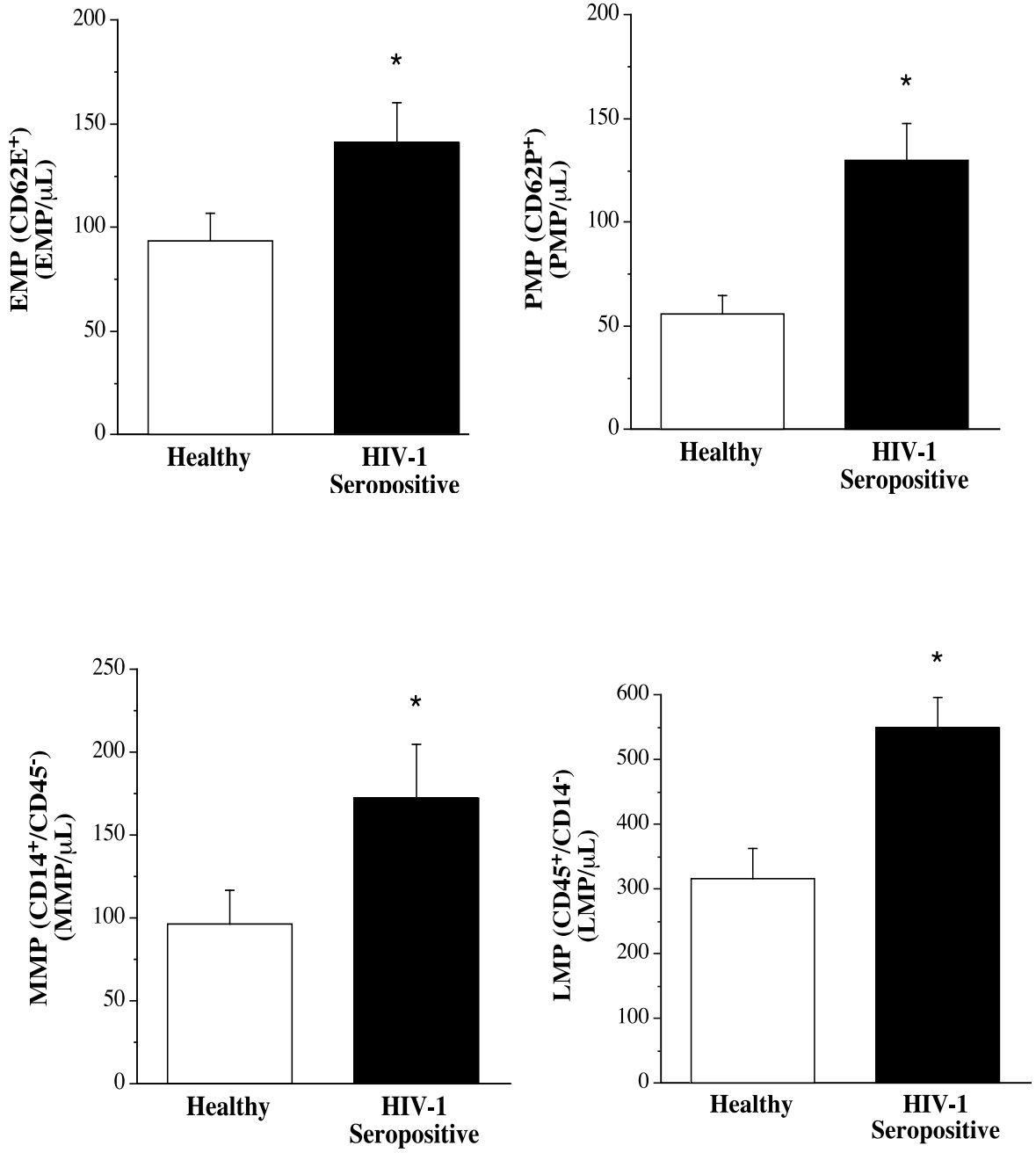

Figure 1. Circulating concentrations of endothelial (EMP)-, platelet (PMP)-, monocyte (MMP)- and leukocyte (LMP)-derived microparticles in ART treated HIV-1-seropositive men and healthy men. Values are mean±SEM. **P<0.05*

Endothelial Inflammation

The effect of microparticles on HUVEC cytokine release, expression of NF-κB p65 and p-NF-κB p65 (Ser536) and miR-146a is shown in Figure 2. Microparticles from HIV-1-seropositive men induced greater (P<0.05**)** release of IL-6 (121.4±6.3 vs 101.9±5.3 pg/mL**)** and IL-8 (258.3±14.5 vs 191.4±12.5 pg/mL) from HUVECs into the media compared with the microparticles from healthy men. Intracellular expression of total NF-κB p65 was not significantly different between the cells treated with microparticles from the HIV-1-seropositive or healthy men (2.71±0.35 vs 2.35±0.37 AU). However, the level of p-NF-κB p65 (Ser536) [active NF-κB] was ~120% higher (P<0.05) in HUVECs treated with microparticles from the HIV-1-seropositive (1.57±0.72 AU) compared with healthy (0.71±0.13 AU) adults. Intracellular expression of miR-146a, a miRNA involved in the inhibition of NF-κB activation, was several-fold lower (P<0.05) in cells treated with microparticles from HIV-1-seropostive men than those from healthy controls (2.80±0.41 vs 6.59±0.72 AU). Expression of miR-181b (1.35±0.20 vs 3.18±0.63 AU) was also lower in the cells treated with microparticles from the HIV-1 infected men. In the overall study population, p-NF-κB p65 (Ser536) expression was positively related to IL-6 (r=0.42; P<0.05) and IL-8 (r=0.41; P<0.05) release and inversely related to miR-146a expression (r=-0.47; P<0.05) (Figure 2). There was no correlation between IL-6 or IL-8 concentrations and miR-181b.

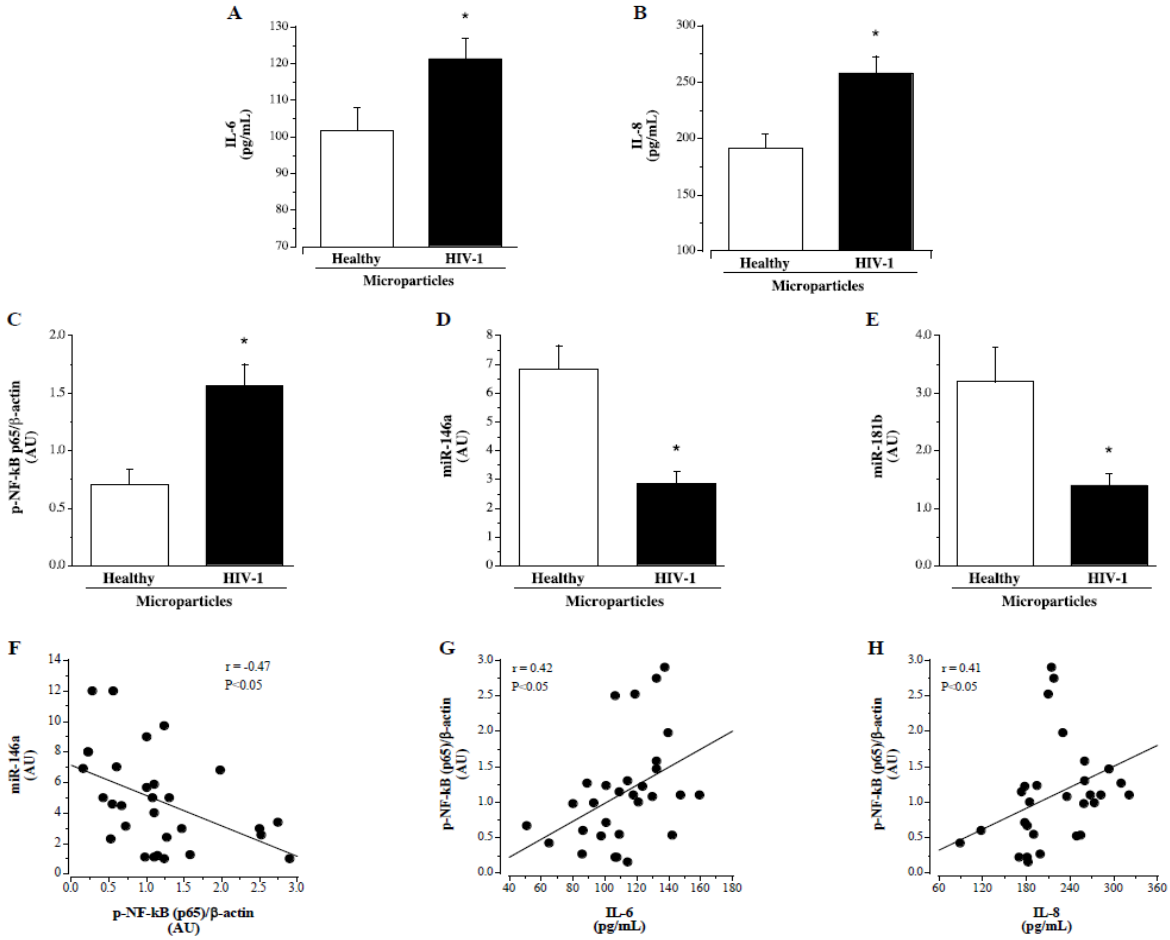

Figure 2. Endothelial cell release of IL-6 (panel A) and IL-8 (panel B) and intracellular expression of p-NF-κB p65(Ser536) (panel C), miR-146a (panel D) and miR-181b (panel E) in response to treatment with microparticles from ART treated HIV-1-seropositive men and healthy men. Relation between miR-146a and p-NF-κB p65 (panel F) in microparticle treated HUVECs. Relation between p-NF-κB p65 and IL-6 (panel G) and IL-8 (panel H) concentrations from microparticle treated HUVECs. Values are mean±SEM. *$P<0.05$*

Endothelial Oxidative Stress

Microparticles from HIV-1-seropositive men prompted greater (~40%; $P<0.05$) cellular reactive oxygen species (ROS) production compared with microparticles from healthy men (147±13 vs 107±9 % of control) (Figure 3). Moreover, the expression of ROS-induced chaperone protein, Hsp70, was also significantly higher in the HUVECs treated with the microparticles from HIV-1-infected men (2.81±0.57 vs 1.36±0.13 AU).

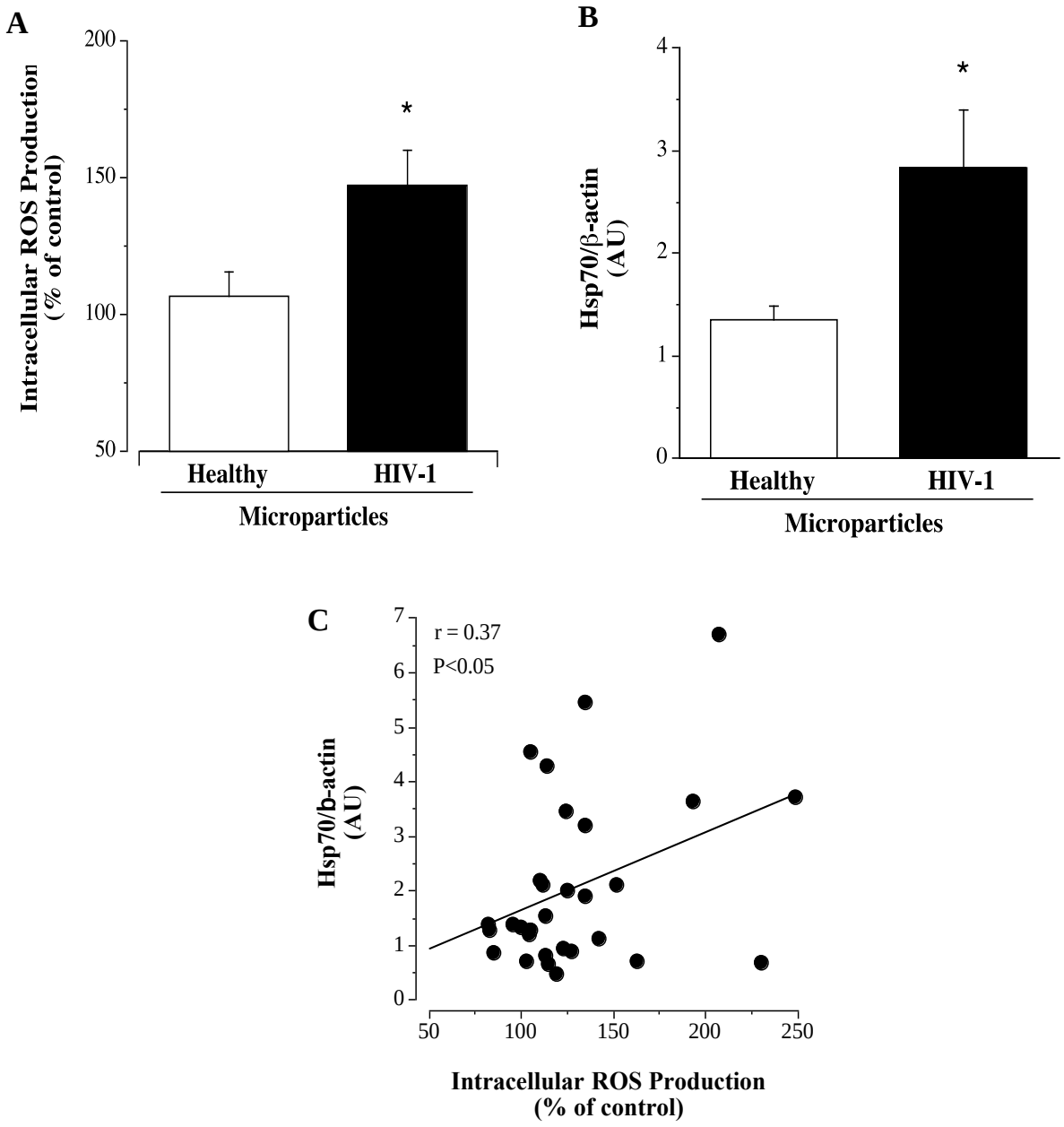

Figure 3. Endothelial cell reactive oxygen species production (panel A) and intracellular Hsp70 expression (panel B) in response to microparticles from ART treated HIV-1-seropositive men and healthy men. Relation between Hsp70 and reactive oxygen species production (panel C) in microparticle treated HUVECs. Values are mean±SEM. **P<0.05*

Endothelial Senescence

Endothelial cell senescence as well as cell expression of SIRT-1 and miR-34a are shown in Figure 4. Microparticles from the ART-treated HIV-1-seropositive adults induced greater endothelial cell senescence. The percentage of SA-β-gal staining cells was significantly higher in HUVECs treated with microparticles from HIV-1-seropositive (36±1 %) compared with healthy (27±1 %) adults. HUVEC expression of SIRT-1 was ~50% lower (4.17±0.54 vs 6.17±0.71 AU; $P<0.05$); whilst, the expression of miR-34a was ~90% higher (3.45±0.51 vs 1.83±0.39 AU) in cells treated with microparticles from HIV-1-seropostive versus healthy men. SIRT-1 expression was strongly and significantly inversely correlated ($r=-0.75$) with miR-34a expression.

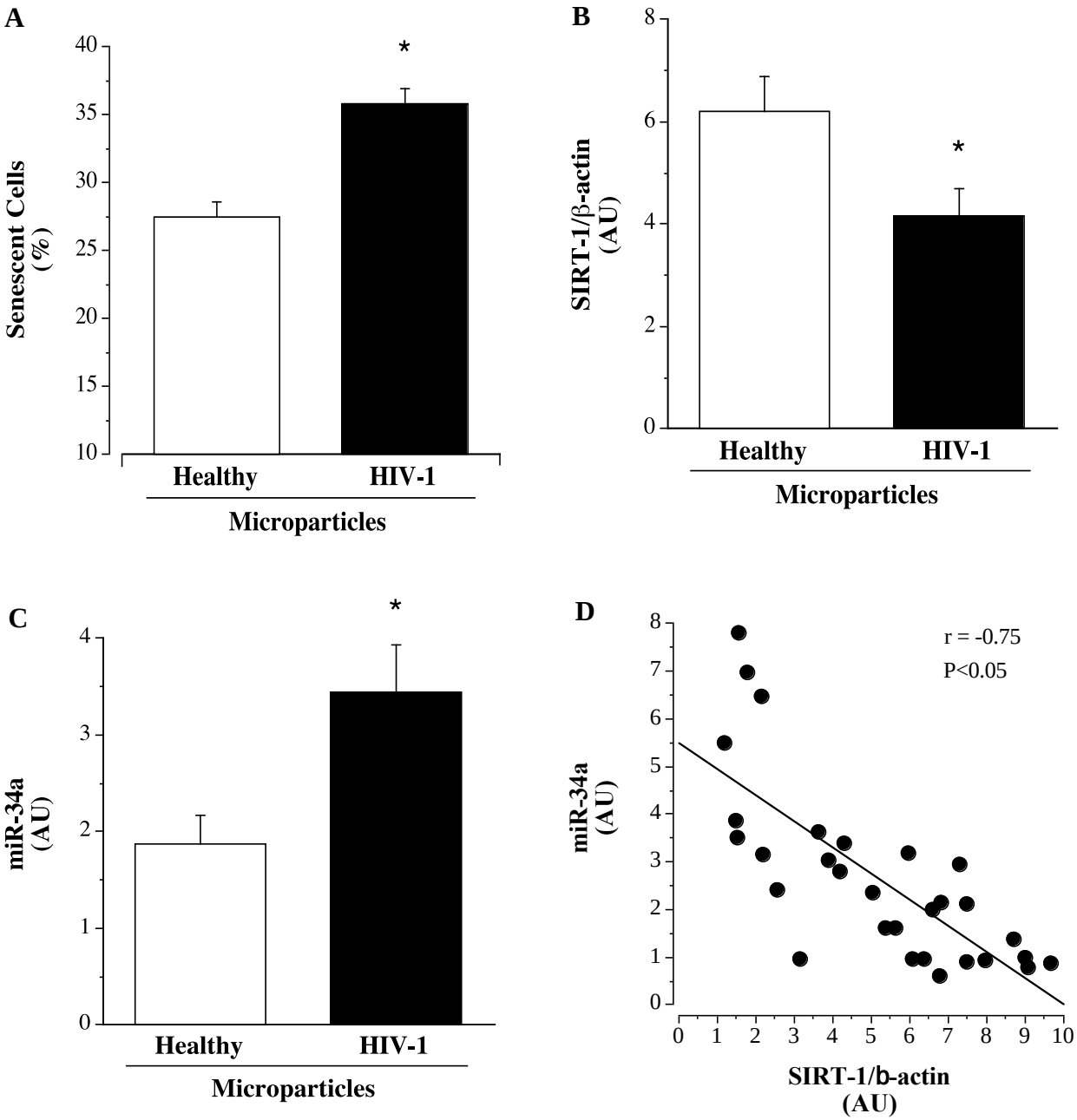

Figure 4. Endothelial cell senescence (panel A) and intracellular SIRT-1 (panel B) and miR-34a (panel C) expression in response to treatment with microparticles from ART treated HIV-1-seropositive men and healthy men. Relation (panel D) between cellular miR-34a and SIRT-1 expression in microparticle treated HUVECs. Values are mean±SEM. **P<0.05*

Endothelial Apoptosis

Endothelial cell expression of caspase-3, active caspase-3 and miR-Let-7a are shown in Figure 5. Microparticles from HIV-1-seropositive men induced significantly higher (~50%) expression of total caspase-3 (2.50±0.21 vs 1.66±0.13 AU) and active caspase-3 (0.92±0.09 vs 0.64±0.08 AU) than microparticles from healthy men. Higher levels of caspase-3 expression was accompanied by reduced expression of miR-Let-7a in the HIV-1-seropositive (3.10±0.40 AU) compared with healthy (7.5±1.1 AU) microparticle treated cells. Total caspase-3 levels were inversely related ($r=-0.48$; $P<0.05$) with miR-Let-7a expression.

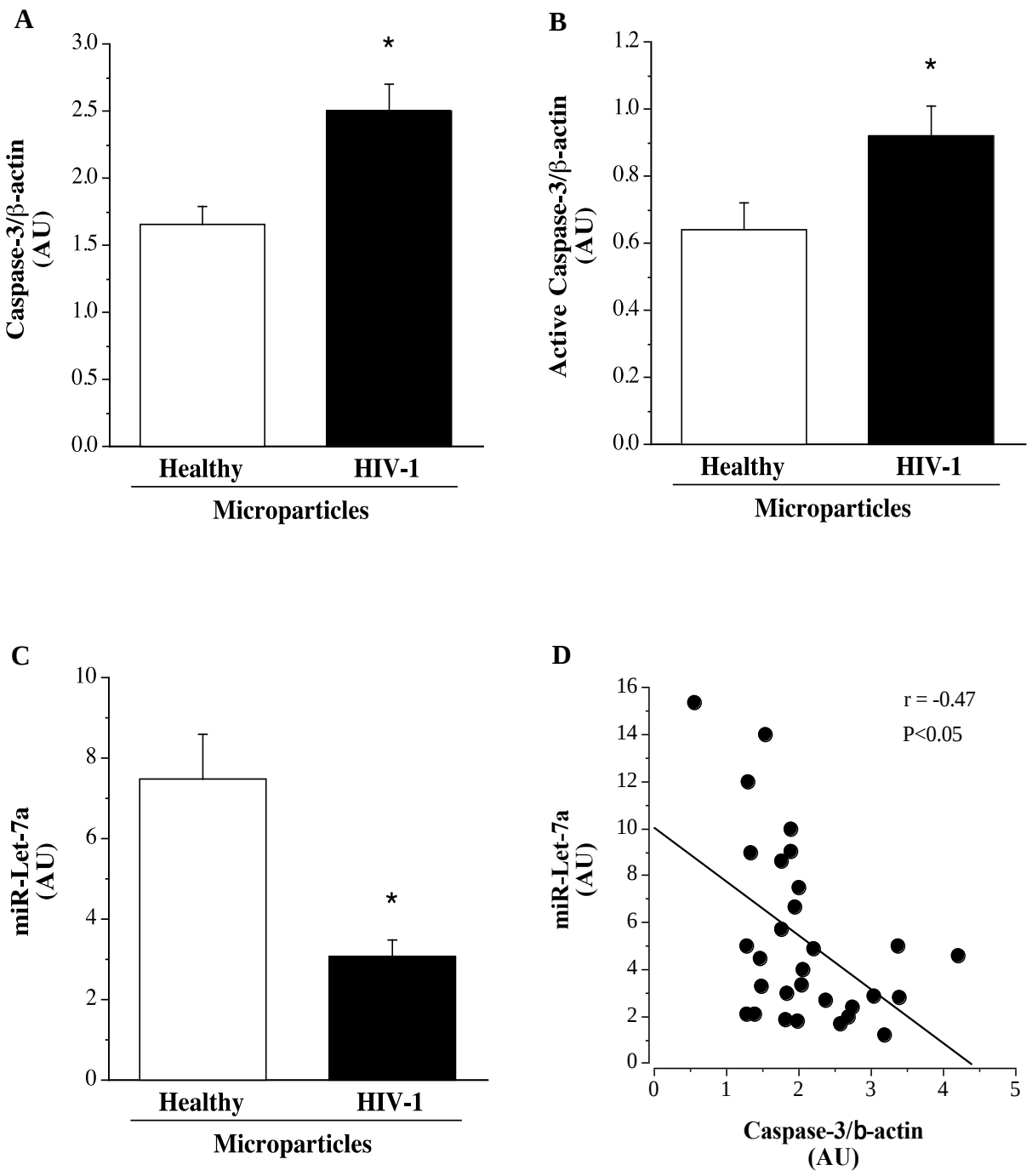

Figure 5. Endothelial cell expression of caspase-3 (panel A), active caspase-3 (Asp175) (panel B), and miR-Let-7a (panel C) in response to treatment microparticles from ART treated HIV-1-seropositive and healthy adult men. Relation (panel D) between intracellular miR-Let-7a and caspase-3 expression in microparticle treated HUVECs.

Endothelial Nitric Oxide Synthase Expression

Cellular expression of endothelial nitric oxide synthase (eNOS), p-eNOS (Ser1177), miR-21, miR-126 and miR-155 are shown in Figure 6. Total eNOS expression was not significantly different between cells treated with microparticles from HIV-1-seropositive (1.65±0.20 AU) and healthy (1.57±0.35 AU) adults; however, p-eNOS (Ser1177) expression was ~40% lower ($P<0.05$) in the cells treated with microparticles from HIV-1 infected men (1.41±0.21 vs 2.42±0.26 AU**)**. Along with eNOS activity, cellular expression of miR-21 (0.63±0.11 vs 1.08±0.16 AU) and miR-126 (2.35±0.36 vs 3.90±0.71 AU) was lower in cells treated with microparticles from HIV-1-seropositive compared with healthy men. There was no difference in the cellular expression of miR-155 between HIV-1 (0.46±0.11 AU) and healthy (0.59±0.09 AU) microparticle treated cells. In the overall study population, p-eNOS (Ser1177) was inversely associated with miR-21 ($r=-0.46$; $P<0.05$) and positively associated with miR-126 ($r=0.38$; $P<0.05$) expression.

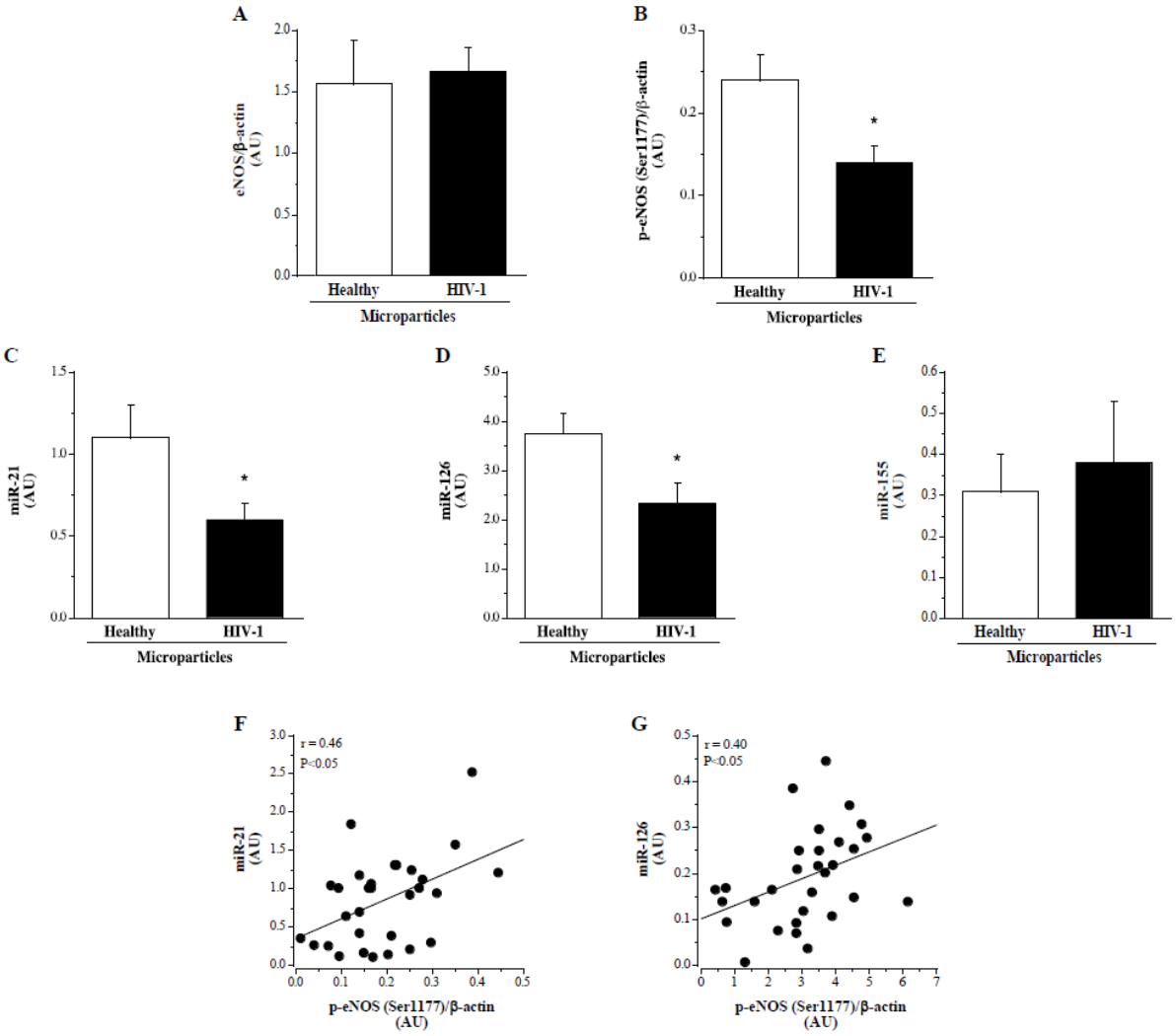

Figure 6. Endothelial cell expression of eNOS (panel A), p-eNOS (Ser1177)(panel B), miR-21 (panel C), miR-126 (panel D) and miR-155 (panel E) in response to treatment microparticles from ART treated HIV-1-seropositive and healthy adult men. Relation between intracellular miR-21 (panel F) and miR-126 (panel G) and p-eNOS expression in microparticle treated HUVECs. Values are mean±SEM. *$P<0.05$

DISCUSSION

The key findings of the present study are as follows: 1) circulating concentrations of EMPs, PMPs, MMPs and LMPs are markedly higher in ART-treated HIV-1-seropositive men compared with their healthy counterparts; and 2) microparticles isolated from ART-treated HIV-1-seropositive men induce a proinflammatory, pro-oxidative, prosenescent and proapoptotic endothelial phenotype. To our knowledge, this is the first study to determine the numerical and functional phenotype of circulating microparticles in HIV-1-infected adults. Elevations in circulating microparticles that are capable of causing deleterious, atherogenic effects on endothelial cell function represent novel factors that could contribute mechanistically to the increased risk and incidence of atherosclerotic vascular disease associated with HIV-1.

Although most eukaryotic cells possess the capacity to release microparticles, clinical interest in circulating microparticles has focused primarily on EMPs, PMPs, LMPs and MMPs because of their principal involvement in the initiation, development and progression of atherosclerotic vascular disease and its clinical consequences [16,36–38]. Indeed, elevations in these microparticles are associated with several cardiovascular pathologies and risk factors including, atherothrombosis, myocardial infarction, ischemic left ventricular dysfunction, unstable angina, diabetes, hypertension and metabolic syndrome [13,13,25,39]. Herein, we demonstrate that the circulating levels of on EMPs, PMPs, LMPs and MMPs are significantly higher (50%-140%) in ART-treated HIV-1-seropositive adults. Although we are the first to report elevations in each of these microparticle sub-types, is not overly surprising considering HIV-1-infection is associated with activation of their parent cells [7,40–42]. Activation of these cells is likely the primary mechanism underlying the elevated circulating concentrations of these specific microparticles [15,36]. Indeed, cellular activation raises p38 activity resulting in cytoskeletal rearrangement and

membrane vesiculation [36]. In addition to reflecting enhanced cellular activation, elevations in EMPs, PMPs, MMPs, and LMPs have been linked to endothelial dysfunction; thus, the importance of circulating microparticles may not simply be based on their concentration but, rather, their potential effects on the vascular endothelium [43–45]. Considering that HIV-1, regardless of ART and independent of other risk factors, is associated with profound endothelial cell activation, inflammation, oxidative stress and apoptotic burden, it is possible that elevations in circulating microparticles may be a contributing factor [4,7,12].

The atherosclerotic process is initiated by endothelial cell activation and inflammation that, in turn, promotes and facilitates the recruitment and intrusion of monocytes and leukocytes into the vascular wall [8]. Endothelial cell release of IL-6 and IL-8 are principal mediators of inflammation and is a precipitating event in atherogenesis [46,47]. In the present study, microparticles from ART-treated HIV-1-seropositive adults significantly upregulated endothelial cell release of IL-6 and IL-8, in vitro. In addition, cellular expression of NF-κB and NF-κB activation were markedly higher in cells treated with microparticles from the HIV-1-infected individuals. NF-κB is the principal transcription factor regulating both IL-6 and IL-8 production and release [47]. Thus, the increase in endothelial cell NF-κB activation in response to the HIV-1-related microparticles likely underlies the enhanced release of IL-6 and IL-8. To determine the potential mechanism(s) underlying the changes in NF-κB we focused on the cellular expression of miR-146a and miR-181b, two miRNAs involved in NF-κB regulation [46,48]. miR-146a quells NF-κB activation by targeting several upstream activators of NF-κB, such as IRAK-1 and TRAF-6 [48]. Reduced expression of miR-146a is associated with increased NF-κB-mediated endothelial inflammation [46,48]. Whereas, miR-181b suppresses NF-κB activity by inhibiting importin-α3, a protein involved in nuclear translocation of NF-κB [48,49]. Both miR-146a and

miR-181b were two-fold lower in cells treated with microparticles from the ART-treated HIV-1-seropositive adults. Collectively these findings demonstrate the profound effects of circulating microparticles from HIV-1-infected individuals on endothelial cell inflammatory processes and suggests a novel mechanism for the increase in vascular inflammation associated with HIV-1.

Oxidative stress often accompanies inflammation and is central to the development of atherosclerosis [50]. While reactive oxygen species (ROS) production is an unavoidable consequence of cellular metabolism and is important for cellular signaling, aberrant ROS production results in DNA and organelle damage as well as protein dysregulation and dysfunction rendering the cell susceptible to disease [50,51]. Herein, we demonstrate that microparticles from ART treated HIV-1-seropositive adults increased endothelial ROS production significantly more (~40%) than microparticles from healthy controls. In addition, cellular expression of Hsp70 was also significantly higher in the cells treated with microparticles from HIV-1-seropositive adults. Hsp70 is a chaperone protein which is expressed constitutively at low levels but is rapidly upregulated in response to ROS induced cellular stress [52] and is considered to be a sensitive and specific indicator of oxidative stress and damage [52,53]. Thus, taken together, it appears that circulating microparticles may play an important role in mediating and promoting oxidative damage to the endothelium that is known to occur with HIV-1 infection [51].

Endothelial cell senescence and apoptosis renders the vasculature highly susceptible to atherosclerosis. Senescent prone endothelial cells exhibit diminished regenerative, angiogenic and vasomotor function [54,55]. In addition, senescent endothelial cells develop a pro-inflammatory senescence-associated (SA) secretory phenotype resulting in the production and release of several cytokines and pro-inflammatory signaling molecules, such as IL-6 and IL-8 [55,56]. In the

present study, microparticles from the HIV-1 infected men accelerated endothelial cell senescence. The percentage of SA-β-gal stained HUVECs was markedly higher in the cell cultures exposed to microparticles from the ART treated HIV-1-seropositive men than those from the healthy men. The mechanisms underlying the prosenescent effects of the HIV-1-related microparticles appear to involve dysregulation of key protein and miRNA critical to anti-senescent cellular signaling [55–57]. Indeed, SIRT-1 is an NAD^+-dependent histone deacetylase which inhibits the activity and expression of proteins associated with cellular senescence and apoptosis [57]. It is well established that reduced cellular expression of SIRT-1 is a causal characteristic of a prosenescent cellular phenotype. Herein, we demonstrate that the increase in senescence in the cells treated with microparticles from the HIV-1-infected men may be due, at least in part, to reduced SIRT-1 expression. Further, consistent with suppressed SIRT-1, miR-34a expression was significantly higher in the HIV-1-microparticle treated cells and was inversely related ($r=-0.75$; $P<0.05$) to SIRT-1 levels. miR-34a targets the 3'UTR of SIRT-1 mRNA resulting in translational repression [57]. Prosenescent effects of HIV-1-related circulating microparticles suggests a novel mechanism contributing to accelerated vascular disease associated with HIV-1.

With regard to apoptosis, HUVECs treated with microparticles from the HIV-1-seropositive men exhibited markedly higher total caspase-3 and active caspse-3 protein expression than the cells treated with microparticles from healthy adults. Intracellular concentration of caspase-3, particularly active caspase-3, provides specific biological insight into the apoptotic tendency of a cell [58]. HIV-1-infection is known to escalate cellular apoptosis through various mechanisms that ultimately heighten caspas-3 activity [59,60]. For example, we have shown that HIV-1 proteins gp120 and Tat induce a proapoptotic endothelial phenotype by

increasing intracellular caspase-3 activity [60]. Circulating microparticles may represent another pathway facilitating the cellular apoptotic effects of HIV-1. Consistent with this notion is the finding that the expression of miR-Let-7a was significantly lower in cells treated with microparticles from the HIV-1-seropositive adults compared with microparticles from the healthy adults. miR-Let-7a directly targets caspase-3 mRNA as the 3'UTR of the caspase-3 gene perfectly matches the seed sequence of miR-Let-7a facilitating mRNA translational repression and/or degradation [61]. miR-Let-7a has been shown to suppress drug-induced apoptosis in cells directly via its effect on caspase-3 expression [61,62]. Indeed, we observed a strong inverse relation between cellular caspase-3 and miR-Let-7a expression. It is important to acknowledge that the noted deleterious effects of HIV-1-related microparticles on endothelial cell function are not occurring in isolation. Changes in the inflammatory and oxidative state of cells treated with microparticles from the HIV-1-seropositive men can also affect caspase-3 activity [51,62–64]. Regardless of the mechanism, the proapototic endothelial effects of circulating microparticles associated with HIV-1 provides additional understanding regarding the detrimental vascular effects of the virus.

Endothelial nitric oxide synthase (eNOS) activity and NO generation is critical to endothelial cell homeostasis [8,55,65–67]. A seminal finding of the present study, that is congruent with our cellular results, is that microparticles from HIV-1-seropositive adults diminish NOS activation. Reduced eNOS activation is, arguably, the most prominent factor in endothelial dysfunction and, in turn, atherogenesis [55,68]. Given the importance of eNOS activation to endothelial vasodilator function, it is not unreasonable to suggest that circulating microparticles may contribute mechanistically to impaired endothelial vasodilation observed in HIV-1-infected individuals [12,69]. Interestingly, eNOS activation and not eNOS protein expression was affected

by the HIV-1-related microparticles. The cause for the specificity of the microparticle effect on eNOS is not clear. However, the observed changes in the cellular expression of miR-21, miR-126 and miR-155, miRNAs that directly influence eNOS expression and activation, provide some insight. In line with suppressed eNOS activation, expression of miR-21 and miR-126 was significantly lower in the cells treated with microparticles from the HIV-1-seropositive men compared with microparticles from the healthy men. miR-21 suppresses PTEN which is a negative regulator of eNOS activation [65]; whereas, miR-126 protects the activity of the PI3K/AKT/eNOS signaling pathway and, in turn eNOS activation [66]. Of note, miR-155 was not altered by the HIV-1-related microparticles. miR-155 directly targets eNOS mRNA reducing its expression, no change is miR-155 is concordant with unaffected eNOS expression [70]. Microparticle-induced reduction in eNOS activation provides a novel mechanism for HIV-1-related vascular dysfunction and accelerated atherosclerosis.

There are a few experimental considerations regarding the present study that deserve mention. Firstly, inherent with all cross-sectional human studies we cannot dismiss the possibility that genetic and/or lifestyle behaviors may have influenced the results of our group comparisons. However, to minimize the effects of other lifestyle behaviors, all subjects were sedentary, non-obese and non-smokers. Moreover, all subjects were carefully screened to eliminate the confounding effects of clinically overt metabolic and/or cardiovascular disease. Secondly, since we did not inhibit or counteract the observed microparticle effects on miRNA expression; thus, we are unable to discern the exact contribution of each miRNA on either their specific cellular target or functional outcome. Thirdly, although all of the HIV-1 seropositive men were on their first ART-regime, the regimens were not uniform across the subjects. Our sample size is not sufficient to determine whether differences exist between ART-regimens and

microparticle number and function. Finally, our study involved only men. Although we previously demonstrated that circulating EMPs do not differ in healthy middle-aged men and women, we should not assume similar numerical and functional circulating microparticle signature and phenotype in HIV-1-seropositive women. Especially considering that HIV-1-infected women have increased rates of acute myocardial infarctions and ischemic strokes compared with HIV-1-infected men[71].

In conclusion, the results of this study demonstrate that circulating concentrations of cell-derived microparticles (EMPs, PMPs, MMPs and LMPs) are elevated in ART-treated HIV-1-seropositive men. In addition, microparticles from ART-treated HIV-1-seropositive men adversely affect endothelial cells; promoting endothelial cell inflammation, oxidative stress, senescence and apoptosis as well as reducing eNOS activation. It is well established that these changes in endothelial cell phenotype are highly proatherogenic and prothombotic [8,9,50,54,72]. Circulating microparticles represent novel mechanistic mediators of the atherogenic vascular phenotype associated with HIV-1 and viable targets for therapeutic intervention.

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CHAPTER 5

CONCLUSION

The primary new findings of the studies presented herein are as follows:

1) HIV-1 R5 and X4 gp120 and Tat significantly increase endothelial cell senescence and the endothelial cell expression of specific SA-miRNAs is adversely altered by these proteins, thereby promoting a more senescence prone cellular phenotype. 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.

2) HIV-1 R5 and X4 gp120 and Tat induce EMP release from endothelial cells and R5-, X4- and Tat-induced EMPs confer pathologic effects on endothelial cells by promoting inflammation, oxidative stress, senescence and apoptosis and altering cellular miRNA expression. Thus, HIV-1 gp120 and Tat induced EMPs may contribute to the deleterious effects of HIV-1-related proteins on vascular health.

3) The circulating concentration of endothelial cell-, platelet-, monocyte- and leukocyte-derived microparticles are higher in ART-treated HIV-1-seropositive men. Additionally, microparticles isolated from ART-treated HIV-1-seropositive adults dysregulate miRNA expression and induce a proinflammatory, pro-oxidative, prosenescent and proapoptotic endothelial phenotype and diminish eNOS activation. The elevated levels of these proatherogenic microparticles may contribute to the increased risk and incidence of atherosclerotic vascular disease associated with HIV-1.

Collectively, these studies identify microparticles and miRNA as novel mediators of HIV-1-related endothelial dysfunction and provide mechanistic insight into the increased risk and prevalence of endothelial dysfunction and, in-turn, atherosclerotic vascular disease with HIV-1.

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