Enhanced Pulmonary Arteriopathy in Simian Immunodeficiency Virus–infected Macaques Exposed to Morphine

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Rationale: HIV-associated pulmonary arterial hypertension (PAH) is likely a more prevalent noninfectious complication of AIDS than previously recognized. Furthermore, the majority of HIV-PAH cases occur in individuals with a history of intravenous drug use. In this study we used a simian immunodeficiency (SIV) macaque model and a primary cell-culture system to investigate the association between drug abuse and HIV infection in HIV-PAH development.

Methods: The archival lung tissues from macaques previously used to study the effect of morphine on SIV infection–associated neuropathogenesis were analyzed for pulmonary vascular changes. The direct effect of HIV proteins and illicit drugs was investigated on oxidative stress, survival, and proliferation of human pulmonary microvascular endothelial cells.

Measurements and Main Results: SIVmacR71/17E-infected rhesus macaques treated with morphine (VM group) demonstrated significant pulmonary vascular remodeling, including the presence of early and advanced complex (plexiform) lesions, when compared with either the SIV-infected (V group) or morphine-treated uninfected (M group) macaques. However, both the V (two of five) and VM (two of six) groups included some animals with *Pneumocystis jirovecii* pneumonia. The endothelial cells lining the vessels with medial hypertrophy or initial-stage intimal lesions in lung sections from VM macaques demonstrated an increase in positivity for both terminal dUTP nick-end labeling and Ki67. Oxidative stress–mediated enhanced apoptosis followed by enhanced proliferation of endothelial cells was observed on simultaneous treatment with viral proteins and drugs of abuse compared with either treatment alone.

Conclusions: Our findings suggest that SIV/HIV protein(s) and morphine interact to cause the proliferation of apoptosis-resistant endothelial cells leading to angio-obliteration.

Keywords: apoptosis; proliferation; illicit drugs; HIV proteins; Tat

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AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

HIV and intravenous drug use continue to be global health problems. Both have been identified as common independent risk factors of pulmonary hypertension. However, HIV-associated pulmonary arterial hypertension (PAH) appears more prevalent in patients with the concomitant history of intravenous drug abuse, indicating a possible “multiple-hit” phenomenon. Although opioids are known to be common drugs of abuse and their effects on the vasculature and endothelium have been documented, their interaction with HIV infection in causing pulmonary vascular injury is not well studied.

What This Study Adds to the Field

This study suggests a potential additive effect of morphine abuse on HIV-mediated endothelial injury and pulmonary vascular remodeling using simian immunodeficiency virus–infected macaque model. These findings support the multiple-hit mechanism of PAH and speculate that the consumption of drugs of abuse render HIV-infected individuals more susceptible to the occurrence of PAH.

Infection with HIV continues to be a serious health problem across the world. In developed countries, vascular complications have increased in prevalence as infectious consequences have declined. In this regard, one of the most devastating sequelae is the development of pulmonary arterial hypertension (PAH) in HIV-infected individuals (HIV-PAH). HIV-PAH results in early mortality and serves as an independent predictor of survival (1). Despite antiretroviral therapy, 1 in 200 patients with AIDS still develops HIV-PAH (2, 3). However, the disease may be even more underrecognized than previously described, as recent estimates indicate that 35% of patients with asymptomatic HIV have elevated pulmonary artery pressures (4–6). Intravenous drug use (IVDU), commonly involving opioids such as heroin and morphine, accounts for one-third of all new cases of AIDS in the United States (7) and has been identified as the most common risk factor in individuals diagnosed with HIV-PAH (2, 3, 8). In fact, in one of the earliest reports of six HIV-infected patients with clinical PAH, five were IVDUs (9). In addition, comparison of two different studies of HIV-infected cohorts from the United States suggests a higher incidence of increased pulmonary arterial systolic pressure in the cohort that had a greater percentage of IVDUs (10). Furthermore, our recent study showing enhanced pulmonary vascular remodeling in

HIV-infected lung tissues from IV opioids and/or cocaine abusers (11) indicates that IVDU and HIV-1 potentially act in concert to cause a pulmonary arteriopathy. Although it is clearly evident that HIV-infected patients are at an increased risk for the development of PAH, the mechanisms and the pathogenesis of HIV-PAH remain elusive.

The initial apoptotic injury of pulmonary endothelial cells followed by hyperproliferation of apoptosis-resistant cells (12) is believed to be one cause of severe pulmonary hypertension characterized by complex pulmonary vascular lesions. Morphine, apart from exacerbating endothelial dysfunction in the presence of the HIV protein Tat (13), has been shown to stimulate both apoptosis and proliferation of vascular endothelial cells (14–17). Based on these findings, we hypothesized that simian immunodeficiency virus (SIV)-infected rhesus macaques exposed chronically to morphine would demonstrate an accentuated pulmonary vascular arteriopathy. To test this hypothesis, we analyzed archival lung tissues from macaques used earlier to study the effect of morphine abuse on the neuropathogenesis and immune pathogenesis of SIV infection (18). Here we show enhanced pulmonary arteriopathy with the presence of complex lesions in the SIV plus morphine group compared with either the morphine or SIV group. Our cell-culture studies show both increased apoptosis and proliferation of pulmonary endothelial cells on simultaneous exposure to both morphine and Tat. Some of the results of these studies have been previously reported in the form of abstract (19).

MATERIALS AND METHODS

Macaques

The paraffin-embedded archival lung sections from 11 male SIV-infected rhesus macaques exposed (virus + morphine [VM] group, $n = 6$) or unexposed (virus only [V] group, $n = 5$) to morphine for up to 59 weeks were assessed for histological evidence of pulmonary vascular lesions and compared with morphine-exposed uninfected control animals (morphine [M] only group, $n = 5$) (18). Briefly, 26 weeks of morphine injections (2.5 mg/kg, intramuscular, four times daily) were administered to macaques in the M and VM groups, and sham injections of saline were given to macaques in the V group. After this treatment period, the animals in the VM group and V group were inoculated with SIVmacR71/17E in the bone marrow, followed by continuation of morphine injections in the M and VM groups and saline injections in the V group. Animals were killed at the end of 31 weeks after the viral inoculation; however, two macaques in the VM group (C065 and D264) and one macaque in the V group (C089) that developed clinical disease between 13 and 16 weeks had to be killed sooner for collection of tissues. No hemodynamic measurements were obtained on any of the macaques. For more details, please refer to previous publication by Marcario and colleagues (18). Hematoxylin and eosin (H&E), Verhoeff von Gieson (VVG), Masson's trichrome, and immunohistochemical staining of paraffin-embedded lung sections was performed as previously described (20). See the online supplement for further details on histochemistry and *in situ* terminal dUTP nick-end labeling (TUNEL) assay.

Cell Culture and Treatments

Human primary pulmonary microvascular endothelial cells (HPMVEC) were treated with HIV-Tat 1-72 (25 ng/ml), Nef (10 ng/ml), or gp-120_{CM} (100 ng/ml) in the presence or absence of morphine (10^{-6} M) for indicated time intervals (see online supplement).

Apoptosis and proliferation were determined by Cell Death Detection ELISA and Cell Titer 96Aqueous One Solution Cell Proliferation Assay, respectively (see online supplement for further details).

Hydrogen peroxide (H_2O_2) and superoxide radical (O_2^-) generation were examined using Amplex red and superoxide dismutase (SOD)-inhibitable cytochrome-c reduction assays, respectively. Further details and statistical analysis are provided in the online supplement.

RESULTS

Histopathological Changes in the Lungs from Morphine-treated and/or SIV-infected Macaques

Enhanced vascular remodeling was found in the VM group macaques compared with macaques in V and M groups, including moderate to severe medial hypertrophy, intimal lesions, endothelial disruption with adventitial thickening or luminal obstruction, and presence of early and complex plexiform lesions (Table 1). The representative H&E image from one of the VM group macaques (C053) with a nearly occlusive neointimal lesion and the VVG image from macaque C065 with the initial stage of plexiform lesion and aneurysm complex lesions are shown in Figure 1. In contrast, only less severe lesions with mild focal, medial, and intimal lesions or adventitial remodeling were observed in two of the five macaques in the V group (Table 1, Figure 1). The complex lesions were not observed in this group. Pulmonary arteriopathy was more advanced in the M group compared with the V group, as almost all of them showed a disrupted endothelial layer with swollen cells and mild to moderate medial hypertrophy. In addition, intimal proliferation was present in some of them (Table 1, Figure 1). One of the M-group macaques (C110) had occasional severe lesions showing partial luminal blocking, as shown in Figure 1. Representative H&E photomicrographs of normal macaque lungs, with no morphine and SIV treatment, are shown in Figure E1 in the online supplement.

Immunostaining for α -smooth muscle actin (SMA), and factor VIII indicated moderate to significant medial hypertrophy of proximal as well as nonmuscular arteries with significant endothelial cell blebbing, swelling, and proliferation leading to the luminal occlusion in the lung sections from the VM group, as shown in the representative image from C095 macaque (Figure 1, lower panel). Blebbing and swelling of endothelial lining as well as medial hypertrophy were common in macaques from the M group. In contrast, an almost normal endothelial lining with mild medial hypertrophy was observed in SIV-infected macaques from the V group (Figure 1).

Remarkably, macaque C088, from the VM group, had significant vascular remodeling with various advanced pulmonary vascular lesions. Changes such as eccentric neointimal and obliterating plexiform lesions composed of hyperchromatic cells around slit-like vascular channels were noted (Figure 2A). As expected, we found accumulation of collagen within the plexiform lesions as well as in adventitia (Figure 2B) of the lesion corresponding to that shown in Figure 2A. Figures 2C–2E show representative VVG-stained images with fully occluded vessels with plexiform/intimal fibrotic and neointimal lesions (Figure 2C), plexiform lesions clearly showing multiple vascular channels formed within an artery (Figure 2D), and presence of lymphomonocytic vasculitis (Figure 2E). Significant inflammation within the wall as shown in the H&E image of longitudinal vessel is noted in Figure 2F. The SMA and factor VIII staining of the lung section of C088 macaque showed nearly complete lumen obliteration of intraalveolar precapillary arteries with factor VIII-positive endothelial cells surrounded by smooth muscle-positive cells (Figures 2E and 2G). Most of the cells surrounding the factor VIII-positive endothelial cells lining the slit-like vascular channels within the complex lesions were positive for both factor VIII and α -SMA (Figure 2F).

Given that inflammation plays a significant role in HIV-PAH (21–23), we stained the sections for CD68, a monocyte/macrophage marker. Mild to moderate inflammation was observed in all macaque samples (Table 1), with evidence of an increased number of macrophages in the lung sections from the VM group compared with the M or V group macaques.

TABLE 1. SUMMARY OF PATHOLOGICAL CHARACTERISTICS OF LUNGS FROM SIMIAN IMMUNODEFICIENCY VIRUS-INFECTED MACAQUES TREATED WITH OR WITHOUT MORPHINE

Monkey ID no.	Group	No. of Weeks: Morphine and/or SIV	Lung Histology	Pulmonary Vascular Remodeling*	CD68
98-C067	M	59	Focal inflammation	Focal bone marrow embolism, mild medial hypertrophy with focal intimal proliferation (+)	1.5+
98-C110	M	34	Vasculitis with infiltration of lymphocytes, eosinophils, and neutrophils	Medial hypertrophy, intimal proliferation, neointimal lesions, occasional near luminal blocking, endothelial blebbing and proliferation (+++)	
98-C097	M	59	Normal	Sporadic medial hypertrophy of proximal vessels with adventitial thickening (+)	1+
98-C070	M	34	Focal inflammation around vessels, vasculitis	Medial hypertrophy of both proximal and distal vessels, endothelial disruption, focal fibrin thromboembolism (++)	
98-C042	M	59	Moderate perivascular cuffing	Mild to moderate medial hypertrophy, intimal proliferation (++)	1.5+
98-C025	V	57	Focal PC pneumonia, focal atelectasis	Sporadic mild medial hypertrophy (-/+)	1.5+
98-C001	V	55	Moderate inflammation, PC pneumonia, and multinucleated giant cells	Mainly adventitial remodeling with focal mild hypertrophy (+)	2.5+
98-D321	V	56	Focal chronic inflammation	None (-)	1.5+
98-D388	V	57	Moderate inflammation	Medial hypertrophy with focal intimal lesions (++)	1.5+
98-C089	V	39	Minimal inflammation	Sporadic mild medial hypertrophy (-/+)	1.5+
98-C053	VM	57	PC pneumonia, inflammation, and chronic bronchitis	Moderate medial hypertrophy of proximal and distal arteries, neointimal lesions with significant endothelial proliferation leading to reduction in lumen size (+++)	2+
98-C065	VM	41	Minimal inflammation	Medial hypertrophy of medium and small vessels, narrowing in lumen with sporadic occlusion of distal arteries, presence of early stage plexiform lesions, endothelial proliferation (+++)	1.5+
98-C088	VM	57	Interstitial pneumonia with extensive parenchymal and perivascular inflammation	Significant medial hypertrophy with neointimal lesions, significant presence of plexiform lesions, complete occlusion of many arteries, lymphoarteritis (+++++)	3+
98-D264	VM	42	Interstitial inflammation, acute pneumonia, diffuse PC pneumonia, and presence of multinucleated giant cells	Moderate medial hypertrophy with significant endothelial swelling or blebbing, adventitial thickening (+++)	3+
98-D276	VM	57	Moderate chronic inflammation including perivascular cuffing	Bone marrow embolism, focal neovascularization, medial hypertrophy, intimal lesions, and increased adventitia (+)	2+
98-C095	VM	56	Minimal to moderate inflammation	Muscularization of distal arteries, sporadic smooth muscle occluded vessels, endothelial disruption, and fibrosis of vessel wall (++)	2.5+

Definition of abbreviations: M = morphine; PC = *Pneumocystis jirovecii*; SIV = simian immunodeficiency virus; V = virus; VM = virus plus morphine.

*Semi-quantitative score for vascular remodeling is shown in parentheses.

Interestingly, increased perivascular inflammation and vasculitis was mainly present in macaques from the M and VM group. In addition, morphine exposure of SIV-infected macaques (VM group) resulted in increased concentrations of MCP-1 and IL-8 in the plasma compared with macaques in the V or M

group (Figures E2A and E2B), with the exception of macaque C-001 of the V group, which had the largest increase in MCP-1 levels associated with an increased number of macrophages and multinucleated giant cells. Although the increase in the VM group was not statistically significant compared with the

Figure 1. Representative photomicrographs showing pulmonary vascular remodeling in macaques from Morphine (left column), Virus (middle column), and Virus + Morphine (right column) groups. Upper panel shows H&E-stained sections (original magnification, $\times 20$), middle panel is VVG-stained sections (original magnification, $\times 40$), and lower panel represents immunostained sections for smooth muscle actin (brown) and factor VIII (red) (original magnification, $\times 60$). Scale bars, 100 μm . Lung section from macaque C053 with eccentric neointimal lesion, from macaque C065 with plexiform (arrow) and aneurysm lesions (arrowheads), and from macaque C095 with endothelial swelling and proliferation from Virus + Morphine group are shown. H&E section from C110 macaque of Morphine group shows nearly occluded vessel (arrow). H&E = hematoxylin and eosin; VVG = Verhoeff von Gieson.

Figure 2. Main histopathological features in C088 macaque from Virus + Morphine group. H&E (A), Masson's trichrome (B), and VVG (C) staining, showing fully occluded plexiform (arrow), eccentric neointimal (arrow-head), and occluded intimal (*) lesions. Note the perivascular inflammation near the lesion in H&E section (A). (D) Cross-section of plexiform lesion showing multiple vascular channels formed within an artery. (E) VVG and (F) H&E sections showing lymphomonocytic vasculitis. Alpha-smooth muscle actin (α -SMA) (G, H) (brown) and factor 8 staining (H, I) (red) shows endothelial cells surrounded by smooth muscles nearly occluding the lumen of the vessel (G, H). Cells within the advanced stage plexiform lesions were also positive for both α -SMA and factor VIII (I). Scale bars, 100 μ m. H&E = hematoxylin and eosin; VVG = Verhoeff von Gieson.

V group, the increased infiltration of macrophages and the higher MCP-1 and IL-8 levels in the lungs were associated with increased vascular remodeling in the VM group. Specifically, the highest levels of MCP-1 and IL-8 in the VM group were detected in macaque C088, which developed severe vascular remodeling with the presence of complex plexiform lesions. Other prominent findings in the VM group included the presence of *Pneumocystis jirovecii* pneumonia (PCP) in two and interstitial pneumonia in one of six macaques (Table 1). However, the presence of foamy alveolar exudates caused by PCP was also seen in two of five macaques of the V group.

Enhanced Endothelial Apoptosis in Morphine-treated SIV-infected Macaque Lungs

Assuming that endothelial injury is the initiating step of vascular remodeling in the animal model of PAH (24–26), we next sought to examine endothelial cell death in the pulmonary arteries of the macaques. As shown in the representative image from the macaque in the M group (Figure 3), endothelial cells were more frequently positive for TUNEL staining, whereas only a small number of endothelial cells lining the pulmonary arteries of the V group macaques were found to be apoptotic. Macaques from the VM group had strong TUNEL-positive staining in large number of endothelial cells, lining both the distal and proximal pulmonary arteries (Figure E3a). Interestingly, endothelial cells

from vessels with the early-stage plexiform lesions with bud-like protrusions in the lumen were TUNEL positive (Figure 3), whereas the advanced-stage fibrotic plexiform lesions with complete occlusion by endothelial cells did not show positivity for TUNEL staining (Figure E3b). Consistent with the TUNEL assay findings, analysis of sE-selectin protein, an endothelial injury marker in the plasma, demonstrated a trend toward up-regulation of sE-selectin levels in the VM group macaques compared with macaques in the M and V groups (Figure E2c).

Increased Proliferation in the Pulmonary Endothelium of Morphine-treated SIV-infected Macaque Lungs

We next assessed the proliferation status of endothelial cells in pulmonary arteries of all macaques by immunohistochemistry for Ki67. Endothelial cells lining pulmonary arteries with or without mild hypertrophy in the V group macaques did not show any positivity for Ki67. A large number of surrounding inflammatory cells were noted to be positive for Ki67 (Figure 3). Similarly, inflammatory cells were positive for Ki67 in the M group macaques, but staining in the endothelial cells lining the remodeled vessels was absent in vessels showing medial hypertrophy. However, pulmonary arteries with medial hypertrophy and neointimal lesions of VM group macaques were frequently lined with Ki67-positive endothelial cells, as shown in Figure 3. Similarly, Ki67 positivity was observed in endothelial cells within

Figure 3. Increased apoptosis and proliferation in endothelial lining of vessels from simian immunodeficiency virus-infected macaques exposed to morphine when compared with macaques from Morphine or Virus alone groups. TUNEL (upper panel, blue) and Ki67 (lower panel, blackish brown) staining in Morphine, Virus, and Virus + Morphine group. Scale bars, 100 μ m. TUNEL = terminal dUTP nick-end labeling.

shown are mean ($\pm$ SD) of three independent experiments, in triplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with untreated control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared with morphine treatment alone; \$ $P < 0.01$, \$\$ $P < 0.001$ compared with Tat treatment alone; † $P < 0.001$ compared with Nef treatment alone; @ $P < 0.01$ compared with gp120 treatment alone.

the completely or partially occluded arteries in the VM group and in inflammatory cells surrounding the lesion and in the lumen (Figure E3c).

Morphine Exposure Increases the HIV Protein-mediated Initial Cell Death of Pulmonary Endothelial Cells

We next analyzed the apoptotic effect of the viral proteins HIV-Tat, Nef, and gp120_{CM} in HPMVECs in the absence or presence of morphine using an oligonucleosome cell death ELISA. As shown in Figure 4A, treatment with either morphine or viral protein alone for 24 hours caused apoptosis when compared with untreated cells. Furthermore, combined treatment of cells with morphine and viral protein resulted in a synergistic increase in the number of dead cells when compared with morphine or corresponding viral protein treatment alone. In addition, a significant increase in cell death after the combined treatment with HIV-Tat and morphine was observed at all time intervals tested when compared with either treatment alone (Figure 4B). A maximum generation of oligonucleosomes was observed at Day 3 post treatment. Exposure of HPMVECs to other commonly used

illicit drugs such as cocaine (10^{-6} M) and methamphetamine (10^{-4} M) also resulted in significant increase in cell death (Figure E4). A relatively higher rate of apoptosis was observed after treatment with methamphetamine compared with morphine or cocaine treatment alone. However, the methamphetamine-mediated synergistic increase of oligonucleosome formation in the presence of Tat (Figure E4) was similar to that obtained after morphine and Tat treatment (Figure 4A).

Increased Proliferation of Endothelial Cells by Simultaneous Exposure to Morphine and HIV Proteins

The presence of Ki67-positive cells in the plexiform lesions and in the endothelial lining of remodeled vessels of the lung sections from SIV-infected morphine-exposed macaques suggested the emergence of proliferative, apoptosis-resistant cells. Therefore, we next examined the direct effect of morphine and viral proteins on the proliferation of pulmonary endothelial cells. As shown in Figure 5A, overnight treatment of quiescent HPMVECs with Tat, Nef, or gp120 in the presence of morphine led to a synergistic increase in the proliferation of HPMVECs compared with either

resulted in increased proliferation of human primary pulmonary microvascular endothelial cells compared with Tat or morphine treatment alone at all days tested. The values shown are mean ($\pm$ SD) of three independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, compared with untreated control; # $P \leq 0.001$ compared with morphine treatment alone; \$ $P \leq 0.05$, \$\$ $P \leq 0.001$ compared with Tat treatment alone. † $P \leq 0.001$ compared with Nef treatment alone, @ $P \leq 0.001$ compared with gp120 treatment alone.

Figure 4. Increased cell death of pulmonary vascular endothelial cells treated with morphine and/or HIV protein. (A) The human primary pulmonary microvascular endothelial cells (HPMVECs) grown in 96-well plates were exposed to Tat (25 ng/ml), Nef (10 ng/ml), or gp120_{CM} (100 ng/ml) overnight in the absence or presence of morphine (10^{-6} M), followed by analysis of apoptotic cells by the Cell Death Detection ELISA kit. (B) Enhancement of cell death after simultaneous exposure of HPMVECs to Tat (25 ng/ml) and morphine (10^{-6} M) compared with either treatment alone when tested at various days of treatment. The data

Figure 5. Increased proliferation of pulmonary vascular endothelial cells treated with morphine and/or HIV protein. (A) Cells were plated on a 96-well plate and next day treated with Tat (25 ng/ml), Nef (10 ng/ml), or gp120_{CM} (100 ng/ml) with or without morphine (10^{-6} M) for 6 days in 0.5% serum-containing medium. The total number of cells in response to various treatments was then measured by CellTiter 96 Aqueous One Solution Cell Proliferation Assay. (B) Simultaneous exposure of Tat (25 ng/ml) and morphine (10^{-6} M)

treatment alone. Growth analysis of morphine and/or Tat-treated cells for varying durations showed a drop in the cell number at Day 2 compared with Day 1 for all treatments (Figure 5B). This was followed by a further increase in cell proliferation at Day 3, with a synergistic increase after combined treatment with morphine and Tat compared with Tat or morphine treatment alone at Day 6. Similar increase in proliferation was also observed in pulmonary smooth muscle cells after treatment with morphine and/or Tat (Figure E5). As shown in Figure E6, exposure of endothelial cells to cocaine or methamphetamine for 6 days also resulted in a significant increase in cell proliferation similar to that after morphine treatment. However, the increase in cell number after the combined treatment of cocaine or methamphetamine in the presence of Tat was less when compared with that observed in the presence of both Tat and morphine treatment (Figure 5A).

Exposure to HIV Protein Plus Morphine Promotes Oxidative Stress and Alters Vascular Endothelial Growth Factor Receptor Activation/Expression in HPMVECs

To characterize a potential mechanism(s) involved in the increased apoptosis and proliferation of endothelial cells after simultaneous exposure to morphine and viral proteins, we first evaluated reactive oxygen species (ROS) generation after treatments. As shown in Figure 6A, combined treatment with Tat and morphine resulted in a significant increase in the generation

of H_2O_2 when compared with either treatment alone. The presence of catalase prevented the morphine- and Tat-mediated increase in H_2O_2 production, whereas no significant changes were observed in the presence of SOD. Cytochrome c reduction assay also revealed significantly higher O_2^- generation on combined treatment with morphine and Tat when compared with either treatment alone (Figure 6B). Pretreatment of cells with antioxidants prevented the morphine- and Tat-mediated increase in apoptosis (Figure 6C) and proliferation (Figure 6D) of endothelial cells as analyzed at Day 1 and Day 6, respectively.

Next we analyzed the expression and activation of the vascular endothelial growth factor (VEGF) receptor 2 (KDR/FLK-1) in HPMVECs in response to morphine and/or Tat treatment for 1 and 6 days. Western blot analysis of cell lysates after 24-hour treatment with morphine and Tat revealed a significant decrease in the KDR phosphorylation without significant alteration in total KDR expression (Figure 7A). Interestingly, exposure to Tat alone resulted in significant elevation in the expression of both phosphorylated and total KDR. In contrast to the 24-hour treatment, a continual 6-day exposure to morphine plus Tat caused significant increase in both phosphorylation and total expression of KDR (Figure 7B).

DISCUSSION

In this report we demonstrate that SIV-infected macaques exposed to morphine developed a severe pulmonary arteriopathy

Figure 6. Involvement of oxidative stress in morphine and Tat-mediated apoptosis and proliferation of pulmonary vascular endothelial cells. (A) Increased H_2O_2 generation from human primary pulmonary microvascular endothelial cells (HPMVECs) on morphine and Tat exposure. Confluent cells treated with morphine (10^{-6} M) and/or Tat (25 ng/ml) in presence or absence of catalase (10 U/ml) and/or SOD (100 U/ml) for 1 hour were then monitored for changes in H_2O_2 production by Amplex red assay. (B) Increase in superoxide generation by HPMVECs in presence of morphine and/or Tat as assayed by SOD-inhibitable cytochrome c reduction. Confluent cells were exposed to morphine and/or Tat at concentrations mentioned above, in presence of 20 μ M acetylated cytochrome c and/or SOD. (C) Prevention of morphine and Tat-mediated endothelial cell death in the presence of antioxidants. HPMVECs grown on a 96-well plate were treated with antioxidants (0.2 mM L-ascorbate, 0.5 mM glutathione, and 3.5 μ M

α -tocopherol) in 0.5% serum containing medium for 30 minutes followed by overnight exposure with morphine (10^{-6} M) and Tat (25 ng/ml). Cell Death Detection ELISA kit was used to analyze apoptosis. (D) Attenuation of morphine plus Tat-mediated enhanced proliferation of endothelial cells in the presence of antioxidants. Cells were pretreated with the antioxidant cocktail for 30 minutes followed by morphine and Tat treatment for 6 days in 0.5% serum-containing medium. The values shown are means ($\pm$ SD) of three independent experiments. * P < 0.01, ** P < 0.001 compared with untreated control; # P < 0.01, ## P < 0.001 compared with morphine treatment alone; \$ P $\leq$ 0.001 compared with Tat treatment alone; Φ P < 0.01, $\Phi\Phi$ P < 0.001 compared with morphine plus Tat treatment; † P < 0.05, †† P < 0.001 compared with catalase-treated control; Δ P < 0.001 compared with SOD-treated control; @ P < 0.001 compared with catalase- and SOD-treated control. H_2O_2 = hydrogen peroxide; SOD = superoxide dismutase.

Figure 7. Alteration in the activation and expression of vascular endothelial growth factor (VEGF)-receptor 2 (VEGFR-2) after exposure of pulmonary endothelial cells to HIV-Tat and morphine. (A) Significant decrease in VEGFR-2 phosphorylation (*left panel*) with no change in total VEGFR-2 expression (*right panel*) after 24 hours of treatment with morphine and Tat. (B) Increase in both phosphorylated (*left panel*) and total VEGFR-2 (*right panel*) after 6-day exposure of human primary pulmonary microvascular endothelial cells to morphine and Tat. Cells grown to 80% confluence were treated with HIV-Tat (25 ng/ml) and/or morphine (10^{-6} M) for 24 hours or 6 days followed by protein extraction and sequential immune-blotting with antibodies specifically directed to phosphorylated or total VEGFR-2 and reprobed with a β -actin antibody. Representative blots (*upper*) and histograms (*lower*) representing the average densitometric analysis of the ratio of P-VEGFR2 or total VEGFR-2 normalized to corresponding β -actin band are shown. Data represents mean ($\pm$ SD) from four or more independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with untreated control.

including the presence of complex, advanced-stage intimal and plexiform lesions similar to those found in humans with severe angioproliferative PAH (25). We furthermore offer both *in vivo* and *in vitro* evidence that morphine potentiates viral protein-mediated apoptosis and proliferation of pulmonary endothelial cells, which may have led to strikingly more pronounced pulmonary vascular remodeling in the SIV-infected macaques exposed to morphine than in macaques exposed to morphine or SIV alone.

Earlier reports on macaques demonstrate pulmonary vascular changes including intimal and medial arterial thickening in both SIV-infected and SHIV-infected macaques (26–28). To explore the effect of morphine and HIV-1 interactions on pulmonary vasculature in this study, we used archival lung tissues from an SIV-infected macaque cohort that was earlier designed to investigate the effect of morphine on the neuropathogenesis of SIV infection. Concordant with previous findings, we also report mild medial hypertrophy with focal intimal changes in some of our SIV-infected macaques. Additionally, morphine administration resulted in mild to moderate medial hypertrophy with endothelial disruption in all of the animals within the group, including the occasional blockade of arterial lumens in one of the macaques (C110). Importantly, the most dramatic histopathological changes, including moderate to severe medial hypertrophy, endothelial disruption, and luminal obstruction with advanced intimal proliferative and plexiform lesions, were found in the morphine-treated SIV-infected group. To the best of our knowledge, this is the first time that advanced and end-stage plexiform lesions characteristic of pulmonary arteriopathy associated with severe PAH have been observed in SIV-infected macaques. As reported earlier, no significant differences were found in the CD4 or CD8 counts or plasma viral load between the V and VM groups (18). However, in other studies

of SIV/SHIV-infected macaques, exposure to chronic morphine had a significant and dramatic effect on viral replication in cerebral tissues, resulting in early mortality (29, 30). Furthermore, no correlation was observed between the plasma viral load/CD4 count and the degree of pulmonary vascular remodeling in these groups, although the C088 macaque, with the most pronounced arteriopathy, had the lowest CD4 counts among all of the animals.

Furthermore, we observed that two macaques from the V group and two from the VM group had PCP pneumonia; however, the V group macaques didn't demonstrate as severe vascular remodeling as the macaques in the VM group. *Pneumocystis* infection after CD4 depletion has earlier been reported to cause pulmonary vascular changes related to PAH in a murine model (31). However, a recent report by George and colleagues (26) on SHIV and SIV-infected macaques did not find any correlation of PC colonization or overt PCP with pulmonary vascular changes. They found the same prevalence of PC colonization in SHIV-infected macaques with or without pulmonary vascular remodeling. In the case of SIV-infected macaques, only 32% showed vascular changes, but overt PCP was observed in 60% of total macaques (26).

Endothelial dysfunction and injury of the pulmonary vasculature are believed to be mechanistically important factors in the development of PAH. We observed a large number of apoptotic endothelial cells in vessels with medial hypertrophy or in the early stage of complex vascular lesions in VM group macaques as determined by TUNEL staining. Interestingly, at the advanced stage of fully occluded plexiform or intimal fibrotic lesions, cells stained TUNEL negative, but they were positive for the proliferation marker Ki67. Previously, investigations have proposed (12, 25, 32) that the endothelial apoptosis is necessary for the subsequent increase in proliferation of endothelial cells. Our findings support this concept and suggest that morphine in

combination with viral proteins may have led to an enrichment of pulmonary arterioles with apoptosis-resistant cells, which then may have led to the increase in the severity of angioproliferative remodeling of the pulmonary vasculature in the VM group macaques.

HIV-associated proteins, such as Nef (27, 33, 34), Tat (35, 36), and gp-120 (37–39), have been implicated in endothelial injury. Recently, findings from our laboratory (40) and by Lund and colleagues (41) demonstrate the development of a pulmonary arteriopathy under the influence of HIV-1 proteins alone, leading to pulmonary hypertension in a noninfectious HIV-transgenic rat model. Tat can promote growth and migration of endothelial cells through production of various growth factors as well as the induction of apoptosis of microvascular endothelial cells via caspase activation (24, 42–45). Like Tat, morphine has also been shown to have a dual action on both proapoptotic and prosurvival signals (15, 17). In the present study, we report that morphine along with SIV-infection or with viral proteins additively or synergistically enhance endothelial apoptosis and proliferation of pulmonary endothelial cells, likely through oxidative stress-mediated pathway(s). In addition, decreased VEGF receptor activation at 24 hours and a significant increase in activation and expression of VEGF receptor after 6 days of morphine plus Tat treatment correlate with the initial apoptosis followed by the increase in proliferation of endothelial cells observed under similar conditions. This is in line with previous findings wherein morphine has been shown to induce ROS-mediated apoptosis of human endothelial cells (14) but also to transactivate VEGF and platelet-derived growth factor- β receptors via binding to its mu-opioid receptors on retinal endothelial cells (46). Furthermore, ROS have been reported to be important components of downstream angiogenic signaling cascades initiated by VEGF receptors (47). Our previous *in vitro* findings also confirm the role of viral proteins in the generation of oxidative stress (40) in pulmonary endothelial cells. Therefore, exposure to both HIV proteins and morphine compounded the oxidative stress signals and activation of receptor kinases, explaining the enhanced apoptosis and angiogenic activation of the pulmonary endothelium.

The presence of complex lesions in VM macaques and enhanced apoptosis/proliferation of pulmonary endothelial cells on Tat and illicit drug treatment further corroborate IVDU as one of the important risk factors in the development of HIV-related PAH (2, 3, 8). Similar to our present findings, we earlier reported a potential additive role of HIV infection and cocaine exposure in the development of pulmonary arteriopathy using a pulmonary vascular cell-culture system and archival human lung tissues (11). Furthermore, stimulant use, including amphetamine, methamphetamine, and cocaine, had earlier been reported to be more likely associated in patients with idiopathic PAH (48).

Inflammation plays a key role in the development of many forms of pulmonary arterial diseases and resultant pulmonary arterial hypertension (21–23). Morris and colleagues recently reported for the first time a link between echocardiographic manifestations related to pulmonary vascular disease and the inflammatory phenotype in HIV-infected individuals (49). Consistent with these observations, we also found significantly increased perivascular cell infiltrates around remodeled vessels in addition to a trend toward up-regulation of proinflammatory mediators such as MCP-1 and IL-8 in the VM group. Both MCP-1 and IL-8 have been shown to be elevated in the plasma of patients with various forms of PAH (50, 51).

Based on the data presented here, we speculate that morphine potentiates the development of advanced pulmonary arteriopathy associated with HIV/SIV infection, lending further support to the

notion that HIV-related PAH is a “multiple-hit” phenomenon. Although we acknowledge that our present macaque study is limited by lack of hemodynamic data and measurements of right ventricular hypertrophy and by a small sample size, this report clearly provides proof of concept that morphine treatment results in enhancement of HIV-associated pulmonary vascular remodeling. Studies using opioid antagonists such as naltrexone may be of interest, to confirm the effect of morphine in the development of HIV-PAH.

Author disclosures are available with the text of this article at www.atsjournals.org.

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