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Platelet-derived growth factor expression in primary pulmonary hypertension: comparison of HIV seropositive and HIV seronegative patients

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Platelet-derived growth factor expression in primary pulmonary hypertension: comparison of HIV seropositive and HIV seronegative patients. M. Humbert, G. Monti, M. Fartoukh, A. Magnan, F. Brenot, B. Rain, F. Capron, P. Galanaud, P. Duroux, G. Simonneau, D. Emilie. ©ERS Journals Ltd 1998.

ABSTRACT: Primary pulmonary hypertension (PPH) is characterized by intimal fibrosis and cell proliferation (including fibroblasts, smooth muscle and endothelial cells) in the distal pulmonary arterial tree. Considerable interest has been generated by recent reports of PPH in human immunodeficiency virus (HIV)-1-infected individuals. Although the lack of evidence for a pulmonary artery infection has suggested that in such cases HIV may act through mediator release rather than by direct endothelial infection, the mechanisms underlying HIV-associated PPH remain poorly defined. Platelet-derived growth factor (PDGF) has the ability to induce smooth muscle cell and fibroblast proliferation and migration. Given these considerations, we have attempted to document a possible role for PDGF in PPH occurring in HIV seropositive and seronegative patients.

Using semiquantitative polymerase chain reaction (PCR), PDGF A-chain messenger ribonucleic acid (mRNA) expression was analysed in surgical lung biopsies from 13 HIV seronegative patients and one HIV seropositive patient, all displaying severe PPH. In parallel, lung samples from two patients with HIV-1-associated PPH were studied by immunohistochemistry and *in situ* hybridization. Results were compared to those obtained in three HIV-1-infected individuals with no pulmonary complication (as demonstrated by clinical, radiological, bacteriological, and necropsy findings) and five control lung biopsies.

As compared to controls, PDGF A-chain mRNA expression is elevated in lung biopsies from patients displaying PPH (p=0.029). In HIV-1-associated PPH, interstitial perivascular cells expressing PDGF A-chain mRNA and protein could be detected by *in situ* hybridization and immunohistochemistry, respectively.

Platelet-derived growth factor expression is elevated in lung biopsies of patients displaying primary pulmonary hypertension. Growth factors such as platelet-derived growth factor may play a part in the initiation and/or progression of primary pulmonary hypertension.

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Several cases of human immunodeficiency virus (HIV)-1-infected patients with primary pulmonary hypertension (PPH) have been reported, raising the question of a causal relationship between these two conditions [1–5]. The lack of evidence for a direct HIV-1 pulmonary artery infection by means of electron microscopy, immunochemistry, deoxyribonucleic acid (DNA) *in situ* hybridization, and polymerase chain reaction (PCR) in two patients displaying HIV-1-associated PPH has suggested that HIV-1 may play a role in these cases through mediator release associated with retroviral infection rather than by direct endothelial infection [3].

Regardless of cause, pulmonary hypertension is characterized by smooth muscle cell proliferation in the distal pulmonary arterial tree [6–9]. Pathologically, PPH is characterized by intimal fibrosis and vessel cell proliferation (including fibroblasts, smooth muscle and endothelial cells). Since an increase in the number of vessel cells is such a prominent part of the pathology of PPH, a better definition of the factors that regulate this proliferation is central to an understanding of its pathogenesis. TUDER and co-workers [8, 9] recently presented evidence of perivascular inflammatory cells in plexiform lesions of PPH, suggesting that production of cytokines and growth factors by these cells may be particularly relevant to PPH pathogenesis.

Platelet-derived growth factor (PDGF) is a disulphide-linked polypeptide comprised of two chains (A and B) that give rise to three dimeric isoforms termed PDGF AA, AB, and BB [10]. PDGF-like molecules are secreted by many cell types including smooth muscle cells, endothelial cells, and macrophages [10]. PDGF has the ability to induce the proliferation and migration of smooth cells and

fibroblasts, and it has been proposed as a key mediator in the progression of several fibroproliferative disorders such as atherosclerosis [11], interstitial pulmonary fibrosis [12], chronic allograft rejection [13] and hypoxic pulmonary hypertension [14]. Moreover, expression of PDGF in HIV-related Kaposi sarcoma *in vivo* suggests paracrine and autocrine mechanisms of tumour maintenance in HIV-infected patients [15, 16].

As a first step to document a possible role for PDGF in PPH, we studied PDGF expression in lung samples from thirteen HIV seronegative patients displaying severe PPH and two HIV-infected patients with PPH. These specimens were compared to lung samples from HIV-infected individuals with no pulmonary complication (as demonstrated by clinical, radiological, bacteriological and necropsy findings) and normal controls. We observed the specific production of PDGF in PPH patients irrespective of their HIV serological status, suggesting a role for growth factors in this condition.

Patients and methods

PPH patients

A total of 13 HIV seronegative patients with severe PPH were included in this study. The PPH population corresponded to patients referred to our institution for lung transplantation. Patients had no evidence of infection, inflammatory disease, cancer, congenital heart disease, chronic thromboembolic disease, chronic parenchymal lung disease, or HIV infection. None of the patients had received immunosuppressive drugs. A complete set of baseline haemodynamic data was obtained from each patient, during right-side heart catheterization performed with a No. 7F triple-lumen floatation thermodilution catheter (Swan-Ganz®; Baxter, Santa Anna, CA, USA) demonstrating severe precapillary pulmonary hypertension. At the time of heart-lung transplantation, a surgical lung biopsy was obtained and immediately frozen in liquid nitrogen for PCR.

HIV-associated PPH

Patient 1 (female, 46 yrs of age, post-transfusional HIV-1 infection known for 2 months with a CD4 cell count of 24 cells·μL^{-1}) presented with a 1 yr history of dyspnoea on exertion class II, as defined by the New York Heart Association (NYHA). The patient did not smoke and did not take appetite suppressants or antimigraine drugs. There was no prior history of pulmonary, cardiac, collagen, hepatic or parasitic disease. A chest radiograph showed cardiomegaly and enlarged pulmonary arteries. Electrocardiogram (ECG) revealed sinus tachycardia and right ventricular hypertrophy. Fibreoptic bronchoscopy (FB) and bronchoalveolar lavage (BAL) were normal. A complete set of baseline haemodynamic data was obtained during right-side heart catheterization performed with a 7F triple-lumen floatation thermodilution catheter, establishing the diagnosis of precapillary pulmonary hypertension (table 1). Pathological examination of a surgical lung biopsy (fig. 1A) demonstrated the absence of pathogens, foreign bodies or thrombotic lesions, and evidenced concentric intimal fibrosis of the muscular arteries, medial hypertrophy, plexiform lesions, and perivascular inflammatory infiltrates. A portion of the specimen was immediately frozen in liquid nitrogen and stored at -80°C for PCR, *in situ* hybridization and immunochemistry.

Table 1. – Haemodynamic data

	Patient 1	Patient 2
Right atrial pressure mmHg	8	5
Mean pulmonary artery pressure mmHg	58	45
Wedge pulmonary pressure mmHg	8	6
Cardiac index L·min^{-1}·m^{-2}	2.4	3.2
Pulmonary vascular resistance mmHg·L^{-1}·min^{-1}·m^{-2}	20.8	12.4
Vasodilator: epoprostenol	+	+

+: significant dilation during short-term infusion of epoprostenol (Flolan®, Glaxo Wellcome), at the dose of 5–10 ng·kg^{-1}·min^{-2} over 30–45 min.Vasodilation was defined as a decrease of total pulmonary resistance of at least 20% of the mean of three baseline measurements.

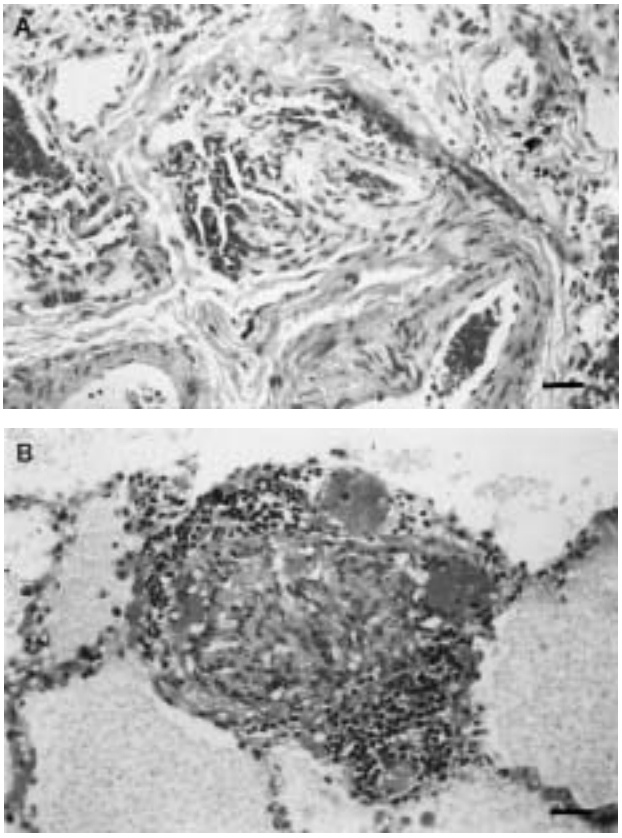

Fig. 1. – Photomicrograph of pathological findings in lung tissue obtained from human immunodeficiency virus (HIV)-1 infected patients with pulmonary hypertension. A) plexiform lesion (patient 1); B) plexiform lesion and pervascular inflammatory infiltrate (patient 2). Haematoxylin eosin saffron stain; internal scale bars 50 μm.

Patient 2 (male, 43 yrs of age, haemophilia A, HIV-1 infection known for 7 yrs with a CD4 cell count of 110 cells·μL^{-1}, and administered 2',3'-dideoxyinosine (ddI)) presented with a 6 month history of dyspnoea on exertion (NYHA class III). The patient presented no risk factor for secondary pulmonary hypertension. Cardiomegaly, enlarged pulmonary arteries, sinus tachycardia and right ventricular

hypertrophy were noted. FB and BAL examination were normal. Right-side heart catheterization confirmed the diagnosis of precapillary pulmonary hypertension (table 1). The patient was treated with oral diltiazem but disease rapidly worsened with untractable right ventricular failure leading to death. Postmortem examination of the lungs showed the absence of pathogens, foreign bodies and thrombotic lesions, and evidenced intimal fibrosis of the muscular arteries, medial hypertrophy, plexiform lesions, and marked perivascular inflammatory infiltrate (fig. 1B). Lung biopsies were fixed in neutral formalin and processed for immunochemistry.

Controls

Five control lung samples taken in unaffected areas from patients undergoing surgery for a lung carcinoma were used in this study. These samples were processed for immunohistochemistry, PCR and *in situ* hybridization. Three HIV-1-infected individuals with no pulmonary complication (as demonstrated by clinical, radiological, bacteriological, and necropsy findings) were studied. These samples corresponded to necropsy material and were used for immunochemistry studies.

Immunochemistry

Immunochemistry studies were performed on lung samples from all HIV-associated PPH cases and controls. The detection of viral antigens was realized using a three-step immunoperoxidase technique with an anti-p24 monoclonal antibody (clone I HIV, final dilution: 1/75; Biosoft, Paris, France), as described previously [17]. PDGF proteins were detected using a goat polyclonal antibody to PDGF recognizing PDGF AA, AB, and BB (anti-AB, Collaborative Research, Le Perray en Yvelines, France) [13], as follows (steps performed at room temperature unless otherwise specified): 0.3% hydrogen peroxide (H_2O_2) in methanol (30 min), phosphate-buffered saline (PBS) (20 min), normal rabbit serum (Dako, Trappes, France) (30 min), primary antibody (anti-AB, 60 min, 37°C, final dilution: 1/2,000), PBS (5 min), biotinylated anti-goat antibody (Vecta Laboratories, Burlingame, CA, USA) (30 min), PBS (5 min), avidin-biotin-peroxydase complex (Vecta, Laboratories) (30 min). Immunoglobulin complexes were then visualized by incubation with 3,3 diaminobenzidine in 0.3% H_2O_2.

Probes for in situ *hyridization*

The PDGF A-chain riboprobe was obtained by subcloning the 362 base pair (bp) Pst 1-BamH 1 fragment of the coding sequence of the PDGF A-chain gene into the T3T7 Bluescript plasmid (Stratagen, La Jolla, CA, USA). The HIV-1-specific probe corresponded to the gag coding sequence of HIV-1 present in a Hind 3-Pst 1 DNA fragment (from bp 75 to bp 995). Sense and antisense ^{35}S-labelled probes were synthesized using T3 and T7 polymerases (Stratagen) according to the manufacturer's recommendations.

In situ *hybridization*

In situ hybridization was performed as described previously [17] on lung samples from Patient 1 and controls. Briefly, slides were postfixed for 20 min in paraformaldehyde (4% in PBS), rinsed in PBS, immersed in 0.1M triethanolamine (pH8) for 5 min at 4°C, and rinsed in 0.1M triethanolamine (pH8) containing acetic anhydride (0.25%) for 10 min at room temperature. The slides were then rinsed and dehydrated in ethanol. Hybridization was performed by adding 30 µL of the hybridization mixture containing 2×10^6 cpm ^{35}S uridine triphosphate (UTP)-labelled ribonucleic acid (RNA) probe. The slides were covered with siliconized glass coverslips and incubated for 16 h at 50°C in a moist box. After hybridization the slides were washed in 5×standard sodium citrate (SSC) 1mM dithiothreitol (DTT) at 42°C for 30 min, and in 50% formamide 2×SSC 1mM DTT at 60°C for 20 min. They were then washed twice for 10 min in NET buffer (0.4M sodium chloride (NaCl), 5 mM ethylenediaminetetra-acetic acid (EDTA), 10 mM Tris buffer (pH7)) at 37°C, and treated with NET buffer containing 20 mg·mL^{-1} ribonuclease (RNAse) (Sigma Chemical Co., St. Louis, MO, USA) for 30 min at 37°C. The slides were then rinsed in NET for 15 min, briefly rinsed in 2×SSC and then 0.1×SSC, dehydrated in 95% ethanol containing 0.3M ammonium acetate, and air dried. For autoradiography, they were dipped into nitroblue tetrazodium (NTB)-2 emulsion (Eastman Kodak, Rochester, NY, USA), and exposed at 4°C for 30–40 days in absolute darkness with desiccant. The slides were developed in Kodak unifix, rinsed, counterstained with Harris haematoxylin, and coverslipped.

Detection of PDGF A-chain messenger (m)RNA by semi-quantitative reverse transcription (RT)-PCR

Semiquantitative RT-PCR was performed as described previously [18]. Total RNA was extracted from surgical lung biopsies from controls and patients displaying severe PPH using the RNAzol technique according to the manufacturer's recommendation (Bioprobe, Montreuil, France). The RNA was treated at 37°C for 30 min with 10 units of RNAse-free deoxyribonuclease (DNAse) (Gibco-BRL, Gaitherburg, MD, USA), then extracted with phenol and chloroform/isoamylic alcohol, and precipitated with ethanol. RNA was then quantified by measuring the optical density at 260nm. RT-PCR was performed using 2 µg of RNA that had been treated for 1 h at 42°C with 50 units of Moloney murine leukaemia virus reverse transcriptase (Gibco-BRL) in the presence of 10 pmoles oligo (deoxy(thymidine) (d[T])12–18) (Gibco-BRL) and 0.5mM of each of the dNTP. For each sample half of the RT product was then processed for PDGF A-chain PCR. To the reaction mixture was added 30 pmol antisense primer (5'-CCTGCCCATTCGGAGGAAGAG-3'), 30 pmol sense primers (5'-TTGGCCACCTTGACGCTGCG-3'), and 2 units of Taq polymerase (Gibco-BRL). The oligonucleotides used are specific for the human PDGF A-chain gene. Each cycle of amplification was at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The products of 35 cycles were subjected to electrophoresis on agarose gels and stained with ethidium bromide. The other half of the RT product was screened for β-actin mRNA using the same procedure

with the following oligonucleotides: 5'-GGTCTCAAACATGATCTGGG-3' and 5'GGGTCAGAAGGATTCCTATG-3' as antisense and sense oligonucleotides, respectively. For both PDGF A-chain and β-actin RT-PCR, samples from patients and controls were processed in parallel. The intensity of the band for PDGF A-chain and β-actin RT-PCR products was determined by scanning using scan analysis software. Results are expressed for each sample as the ratio between PDGF A-chain and β-actin band intensity.

Statistical analysis

Results are expressed as median and range. Data were analysed using a statistical package (Minitab Release 7; Minitab Inc., State College, PA, USA). Mann-Whitney U-test was used for group comparisons. A p-value less than 0.05 was considered significant.

Results

Absence of pulmonary artery HIV-1 infection in HIV-associated PPH

The presence of HIV-1 p24 antigen and HIV-1 gag RNA was analysed in lung samples of patients displaying HIV-associated PPH by immunochemistry and *in situ* hybridization, respectively. Neither HIV-1 p24 antigen nor HIV-1 gag RNA were detected in the wall of pulmonary arteries (not shown).

PDGF A-chain gene expression in lung samples from controls and patients displaying severe PPH

PDGF A-chain mRNA was detected by semiquantitative RT-PCR in lung samples of 14 patients displaying severe PPH (13 HIV seronegative patients and one HIV seropositive patient). The median numbers of copies of PDGF A-chain mRNA relative to β-actin mRNA were greater in lung biopsies from PPH patients than from controls (p=0.029) (fig. 2). PDGF A-chain mRNA expression in the case of HIV-associated PPH was in the same range as that of HIV seronegative patients displaying PPH (fig. 2). When the case of HIV-associated PPH was excluded from statistical analysis, the median numbers of copies of PDGF A-chain mRNA relative to β-actin mRNA were significantly elevated in the thirteen remaining lung biopsies, as compared to controls (p=0.038).

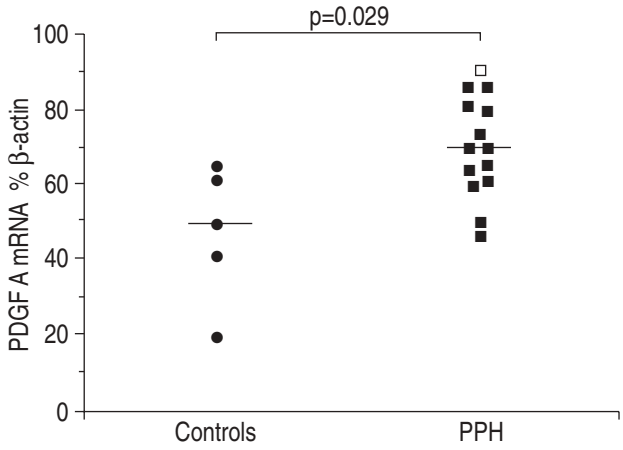

Fig. 2. – Platelet-derived growth factor (PDGF) A-chain messenger ribonucleic acid (mRNA) expression in surgical lung biopsies from controls and patients with severe primary pulmonary hypertension (PPH). PDGF A-chain mRNA expression was measured by semiquantative PCR. Results are expressed as percentages of the numbers of β-actin mRNA copies. Bars represent the median. ●: controls; ■: human immunodeficiency virus (HIV) seronegative PPH patients; ❑: HIV seropositive PPH patient.

PDGF A-chain gene expression in lung samples from controls and HIV-associated PPH

In situ hybridization experiments with the PDGF A-chain probe showed only rare interstitial cells expressing this gene in control lung samples (less than three positive cells per section, data not shown). In contrast, a large number of PDGF A-chain gene expressing cells were evidenced in case of HIV-associated PPH (fig. 3). Most of the positive cells were interstitial cells located at the periphery of small pulmonary arteries (fig. 3). The localization of PDGF A-chain gene expressing cells as well as their morphological characteristics were consistent with an inflammatory cell origin (fig. 3). No signal was detected when using the sense probe (not shown).

Evidence of PDGF-like proteins in HIV-associated PPH lungs

Immunostaining experiments showed few cells labelled with the anti-PDGF polyclonal antibody in control lung samples (not shown). This was in sharp contrast with the

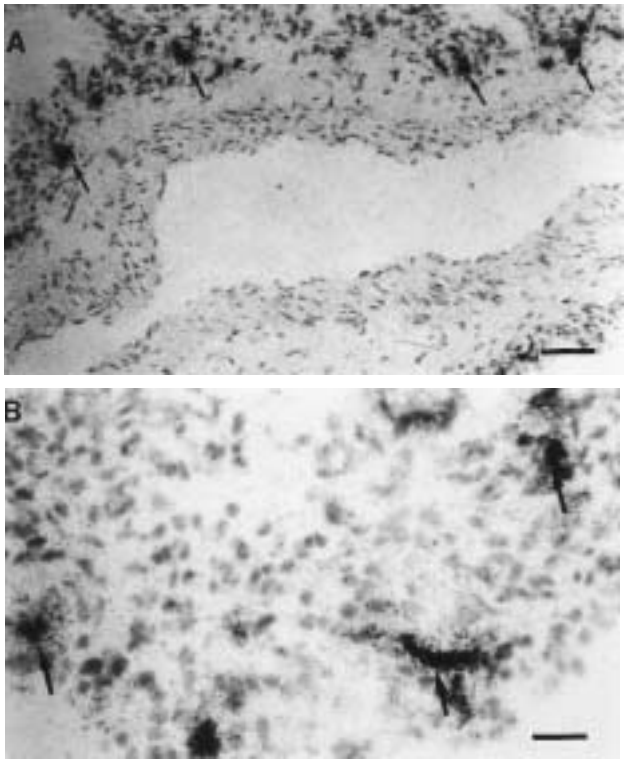

Fig. 3. – *In situ* hybridization with ^{35}S-labelled platelet-derived growth factor (PDGF) A-chain antisense riboprobe. Several PDGF A-chain messenger ribonucleic acid (mRNA) positive primary cells (arrows) are observed in lung tissue obtained from an human immunodeficiency virus (HIV)-1-infected patient with pulmonary hypertension. A: internal scale bar=50 μm. B: internal scale bar=25 μm.

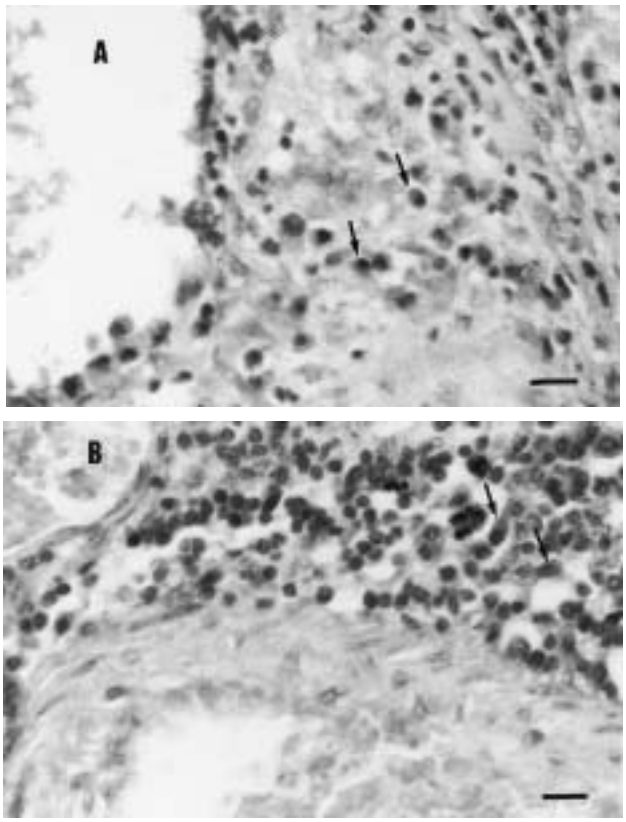

Fig. 4. – Immunohistochemistry with platelet-derived growth factor (PDGF)-specific antibody. Interstitial perivascular PDGF+ positive inflammatory cells (arrows) in lung tissue obtained from human immunodeficiency virus (HIV)-1-infected patients with pulmonary hypertension. A: Patient 1, B: Patient 2. Internal scale bar=30 μm.

immunostaining pattern evidenced in HIV-associated PPH which was characterized by marked PDGF positive (+) perivascular inflammatory cell infiltrates. Similarly to what was shown using *in situ* hybridization experiments, these PDGF-producing cells had a predominantly interstitial distribution (fig. 4).

Discussion

In this study, we demonstrated intrapulmonary PDGF A-chain mRNA expression and PDGF immunoreactivity in two patients displaying HIV-associated PPH. Consistent with the report of Mette *et al.* [3], we have not identified HIV-1 p24 antigen and HIV-1 gag RNA in the pulmonary arteries of these patients. Indeed, it is likely that some HIV-1 complications are not due to direct action of the virus itself but to effects of second messengers such as cytokines and growth factors including PDGF [15]. PDGF receptors are expressed in fibroblasts, smooth muscle cells, and endothelial cells [10], suggesting paracrine and autocrine mechanisms in HIV-associated PPH pathogenesis. Such a mechanism has already been hypothesized in HIV-related Kaposi sarcoma which, similarly, involves increased proliferation of endothelial and smooth muscle cells [15, 16]. Human herpes virus 8 (HHV8) has been recently implicated in HIV-related Kaposi sarcoma pathogenesis [19– 21]. However, this virus does not appear to be involved in PPH pathogenesis, irrespective of its HIV status, as demonstrated by enzyme linked immunosorbent assay (ELISA) and Western Blot analysis of anti-HHV8 serum antibodies (M. Humbert, D. Emilie, T.F. Schulz, unpublished data).

Regardless of the mechanism of its induction during HIV infection, PDGF could play a role in the development of HIV-associated PPH. The predominant perivascular distribution of PDGF-producing cells may be important in this process as the local release of growth factors could explain the specific development of pulmonary artery wall modifications. Since perivascular inflammatory cell accumulation has also been demonstrated in HIV-seronegative patients with PPH [8, 9], local release of growth factors by recruited cells could play a role in the development of pulmonary vessel wall alterations characteristic of this condition. Using semiquantitative PCR we have demonstrated an elevated intrapulmonary PDGF A-chain mRNA expression in lung biopsies from patients with severe PPH, as compared to controls. Therefore, PDGF, in concert with other cytokines and growth factors such as endothelin-1 [22, 23], transforming growth factor-β [24], interleukin-1 [25] and IL-6 [25], is a candidate growth factor in PPH, irrespective of its aetiology.

Interestingly, only a minority of HIV-infected individuals will develop PPH [2, 4], suggesting that displaying PPH in the context of an HIV infection requires some predisposition. A recent report indicates that HIV-associated PPH may reflect a host response to HIV-1 determined by one or more human leucocyte antigen (HLA)-DR alleles located within the major histocompatability complex [26]. We hypothesize that such genetically predisposed individuals may inappropriately produce growth factors in response to HIV, which in turn promote abnormal cell proliferation.

The increased incidence of PPH in patients seropositive for HIV-1 might be due to inappropriate synthesis of growth factors occurring in some genetically predisposed individuals. Interestingly, this might not be unique to HIV-1 as the course of simian immunodeficiency virus infection in macaques can be complicated by the occurrence of pulmonary artery fibroproliferative disorders and perivascular inflammatory cell accumulation in the absence of pulmonary artery wall retroviral infection [27]. PDGF production during HIV-related PPH may thus represent another condition in humans in which a retrovirus induces the deregulation of the synthesis of a growth factor, which in turn is responsible for a complication of the infection. Moreover PPH occurring in HIV seronegative individuals is associated with elevated PDGF production emphasizing that abnormal growth factor production may play a part in all variants of pulmonary hypertension. In HIV seronegative patients with PPH, more studies are needed to identify the source and distribution of PDGF producing cells.

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