



# In Vitro Effects of HIV Proteins, Inflammatory Mediators, HIV-Infected T-Cells, and Pentoxifylline on Endothelial Cell Inflammation

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## Abstract

**Background:** HIV infection is associated with increased serum levels of inflammatory markers, endothelial dysfunction, and cardiovascular events. In a recent pilot trial of HIV-infected patients not requiring ART, we showed that the anti-inflammatory drug pentoxifylline (PTX) improves *in vivo* endothelial function, possibly by inhibiting the leukocyte adhesion pathway. We used cellular models to investigate further the mechanisms roles of HIV infection, inflammation, and PTX on endothelial cell activation.

**Methods:** Recombinant TNF- $\alpha$  and IFN- $\gamma$ , either alone or together with HIV-Tat or gp120, were added to primary human endothelial cells. Additionally, Jurkat T cells (either HIV-infected or not) were co-cultured with endothelial cells. Endothelial cell preparations were collected and analyzed for secretion of the inflammatory chemokine MCP-1, while endothelial cells were harvested for qRT-PCR gene expression analysis of MCP-1 and the leukocyte adhesion molecules IP-10 and VCAM-1. Surface marker evaluations were performed using flow cytometry. Endothelial cells were also treated with PTX, at concentrations achieved *in vivo* in our clinical trial, to assess modulation of gene and protein expression. Significance testing was performed using ANOVA.

**Results:** Gene expression of IP-10 was significantly (P=0.05) enhanced when IFN- $\gamma$  (but not TNF- $\alpha$ ) was combined with either gp120 or Tat. VCAM-1 gene expression was significantly (P<0.001) enhanced by TNF- $\alpha$  (but not with IFN- $\gamma$ ) in combination with either HIV protein. On the other hand, MCP-1 regulation was not affected by these HIV proteins. However, we did find that that endothelial MCP-1 production was significantly upregulated (P=0.0009) when in close contact with HIV-infected Jurkat cells. PTX significantly (P=0.048) reduced this heightened endothelial MCP-1 production.

**Conclusions:** These *in vitro* results demonstrate that HIV proteins, when combined with inflammatory mediators, synergistically and differentially enhance endothelial gene expression of leukocyte adhesion molecules. We further show that PTX can reduce the heightened production of endothelial cell MCP-1 which is induced by cellular contact with HIV-infected T cells. These results extend our understanding of the roles of HIV and systemic inflammatory mediators on endothelial inflammation and may explain the beneficial effects of PTX found *in vivo*.

## Introduction

- Recent data suggest that untreated HIV infection and greater inflammation may promote CV events.
- Endothelial dysfunction is an early step in the development of atherosclerosis and is common in HIV-infected patients.
- We previously demonstrated that pentoxifylline (PTX), an inhibitor of TNF- $\alpha$  production, may improve *in vivo* HIV-related endothelial dysfunction and reduce circulating sVCAM-1 and IP-10 levels.
- We developed *in vitro* models to further assess the roles of HIV infection, inflammation, and PTX on endothelial cell activation and inflammatory molecules (VCAM-1, IP-10, MCP-1).

## Methods and Results

**Gene expression of VCAM-1 and IP-10, but not MCP-1, are synergistically upregulated by combinations of HIV-Tat and gp120 with either INF- $\gamma$  or TNF- $\alpha$  (Figure 1).**

We investigated the possibility that soluble mediators of inflammation synergistically act with secreted viral proteins to induce greater generation of inflammatory cytokines by the endothelium. Based on the literature, we chose to study the interactions between the soluble HIV proteins Tat and gp120 and the inflammatory mediators INF- $\gamma$  and TNF- $\alpha$ , both of which are regarded as key factors in early atherosclerosis. The soluble HIV proteins Tat and gp120 can induce MCP-1 gene expression to levels comparable with TNF- $\alpha$  but fail to further enhance MCP-1 expression when combined with TNF- $\alpha$ . In contrast, VCAM-1 gene production is much stronger when the HIV proteins are combined with TNF- $\alpha$ . On the other hand, IP-10 is induced in synergy when the soluble HIV proteins Tat and gp120 are combined with the inflammatory mediator INF- $\gamma$  but not with TNF- $\alpha$ .

**PTX can reduce MCP-1 chemokine production in response to HIV-infected Jurkat T-cells (Figure 2).**

MCP-1 has been identified as a pro-atherogenic chemokine and has been found in diseased endothelium from HIV-infected patients. Therefore, HIV-infected Jurkat T-cells (CXCR4 strain) were co-cultured in direct contact with endothelial cells. MCP-1 levels were measured by ELISA in the culture supernatants when the endothelial cells were both untreated and pre-treated with PTX (1000nM) for 30 minutes. Pre-treatment with PTX significantly (P=0.048) reduced MCP-1 levels stimulated by HIV-infected Jurkat cells compared to no treatment.

**PTX can reduce VCAM-1 surface expression in response to HIV-secreted proteins and TNF- $\alpha$  (Figure 3).**

Next, we tested if PTX also blocked VCAM-1 production in endothelial cells in response to HIV proteins and TNF- $\alpha$ . We measured the proteins levels of VCAM-1 and IP-10 under exposure with gp120 and TNF- $\alpha$ . Pre-treatment of the endothelial cells with PTX strongly reduced VCAM-1 production but did not affect IP-10 (data not shown).

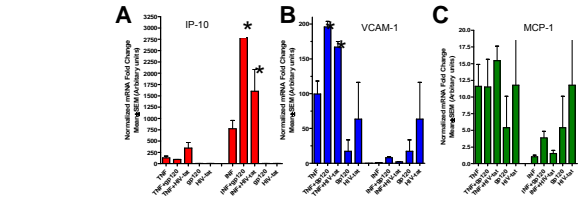


Figure 1. Synergistic upregulation of gene expression of adhesion molecules IP-10 (A) and VCAM-1 (B) but not MCP-1 (C) by interaction between HIV-Tat and gp120 in combination with TNF- $\alpha$  and INF- $\gamma$ . Endothelial cells were incubated for 6 hours with either TNF- $\alpha$  or INF- $\gamma$  alone or in combination with HIV-Tat and soluble gp120 (both 100 ng/ml). Total RNA was isolated and qRT-PCR was performed. Results show fold induction normalized to the endogenous control (elongation factor EF1a) compared to untreated cells. \*p<0.05 cytokine+HIV protein vs cytokine only or vs HIV protein only.

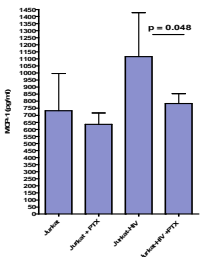


Figure 2. PTX reduces release of MCP-1 from endothelial cells (EC) after contact with HIV-infected Jurkat T-cells. MCP-1 levels in the culture supernatants were analyzed by ELISA. EC were cultured with infected (Jurkat+HIV) or uninfected (Jurkat) T-cells. MCP-1 levels were significantly decreased when EC were pre-treated with PTX (1000nM). Data represent means and SD of 3 experiments.

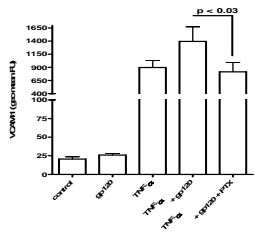


Figure 3. PTX reduces surface expression of VCAM-1 by endothelial cells (EC). EC were treated for 8 hours with TNF- $\alpha$  and/or gp120 with and without pretreatment with PTX (1000nM). Cells were detached and stained with anti-VCAM-1 antibody, coupled to PE-Cy5 (BD Pharmingen), and assessed by using cytofluorometric analysis (BD-Calibur). Data represent means and SD of 3 experiments.

## Conclusions

- VCAM-1 and IP-10, but not MCP-1, endothelial gene expressions are differentially upregulated by combinations of the HIV-secreted proteins gp120 and Tat with either of the inflammatory mediators TNF- $\alpha$  and IFN- $\gamma$ .
- PTX was able to reduce or abolish VCAM-1, but not IP-10, levels in this system.
- Endothelial MCP-1 production was enhanced when in close contact with HIV-infected Jurkat T-cells.
- PTX was able to reduce endothelial MCP-1 production in this system.
- Together, these data suggest that HIV and inflammatory mediators synergistically stimulate the leukocyte adhesion pathway in endothelial cells and thus may contribute to the increased CV risk in HIV-infected patients.
- These *in vitro* models corroborate our *in vivo* data by demonstrating that PTX reduces endothelial activation at the cellular level.

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