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HIV Tat Protein Activates Endothelial Cells through NFκB and MAP Kinase Pathways.

Jason L. Henry
East Tennessee State University

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Jason L. Henry

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Ellen Rasch, Chair
Guha Krishnaswamy, Co-Chair
David Chi
Paul Monaco
Jim Kelley

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ABSTRACT

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HIV infection has been shown to predispose patients to accelerated development of heart disease. One mechanism for this pathology may involve endothelial activation either by HIV itself or by its secreted proteins, gp120 (a viral envelope protein) and tat (a protein that upregulates transcription of viral genes). We have studied the effects of gp120 and tat on signaling and production of inflammatory cytokines by Human Pulmonary Artery Endothelial Cells (HPAEC). HPAEC were stimulated at varying time points with combinations of gp120, tat, and monokines (IL-1β and TNFα). Cell lysate fractions were analyzed for MAP Kinase activity and NFκB activation, and culture supernatants were assayed for inflammatory cytokines (IL-6 and IL-8). The production of IL-6 and IL-8 was significantly enhanced by tat but not by gp120. Both gp120 and tat, however, induced significant morphological changes in HPAEC. The only synergy noted was between high levels of tat and TNFα acting on the production of IL-6. When HPAEC were stimulated with IL-1β and TNFα, peak phosphorylation of p38 MAP Kinase was found at 45 minutes, while NFκB was maximally activated at two hours. Both the ERK1,2 and p38 cascades of MAP Kinase were activated by tat, and an increase in NFκB phosphorylation and translocation was noted. We conclude that the HIV tat protein could be involved in inflammatory changes in endothelium leading to the accelerated development of heart disease in HIV patients.

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ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
APC	Antigen Presenting Cells
DEPC	diethyl pyrocarbonate
ELAM-1	Endothelial Leukocyte Adhesion Molecule 1
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HPAEC	Human Pulmonary Artery Endothelial Cell
IκB	Inhibitor κ B
ICAM	Intercellular Adhesion Molecule
IFN γ	Interferon γ
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-8	Interleukin 8
L-NAME	nitro-L-arginine methyl ester
LTR	Long Terminal Repeat
MAP Kinase	Mitogen Activated Protein Kinase
MCP1	Monocyte Chemoattractant Protein 1
NFκB	Nuclear Factor κ B
PBS	Phosphate Buffered Saline
PKC	Protein Kinase C
PMA	phorbol myristate acetate

RTPCR	Reverse Transcriptase Polymerase Chain Reaction
sTM	Soluble Thrombomodulin
$TGF\beta_1$	Transforming Growth Factor β_1
TNFα	Tumor Necrosis Factor α
VCAM	Vascular Cell Adhesion Molecule
vWF	von Willebrand factor

CHAPTER 1

INTRODUCTION

Since the early days of the HIV epidemic it has been known that there are severe effects of this virus on the immune system. This is shown specifically by the deficit of cell mediated immunity that results from active infection and the resulting opportunistic infections by invading pathogens. As more has been learned about the internal dynamics of the virus and the mechanisms by which it infects cells, we have developed therapies that block the progression of the disease. However, as HIV patients are living longer, they have become the fastest growing cardiovascular disease population in the world.

HIV is a close relative of other lentiviruses, most notably the Simian Immunodeficiency Virus (SIV). Interestingly, SIV infection results in the animal's developing an immune deficiency very much like AIDS in humans. Though they have very diverse genetic sequences, all primate lentiviruses share the common ability to bind to the CD4 receptor of T helper lymphocytes. Though it is far from being clear cut at this point, it is believed that the close association of SIV infected nonhuman primates with humans allowed zoonotic transmission of the virus. This ultimately gave the virus a foothold in the human species (Fields, 1998).

HIV has a mostly typical retroviral life cycle. During the extracellular portion of HIV's life cycle, it travels as a virion. While in this form, it has a protein core bearing two copies of single stranded genomic RNA. This core contains additional material necessary for viral replication such as tRNA primers and reverse transcriptase. The core

is contained in an envelope originally derived from the previous host cell's cell membrane. Before budding, however, viral proteins were inserted into this membrane. These proteins, gp41 and gp120, bind together and form a stalk that facilitates future virion attachment to and entry into a new host cell. This selection is determined by the specificity of gp120 (Fields, 1998). It is generally understood that gp120 recognizes the CD4 receptor on CD4+ cells, but there is also strong evidence that there is further specificity for coreceptors involved. Early stages of HIV infection tend to actively infect macrophages through an affinity toward the CCR5 receptor. There then may be a shift that causes the infection to become more serious. This affinity toward CCR5 is replaced, presumably through genetic drift, by a new preference for the CXCR4 receptor, which is found only on CD4+ cells *in vitro* (O'Brien, 1998). This shift signals the imminent suppression of cell mediated immunity.

When the virion attaches to the CD4 receptor a conformational change in the gp120/gp41 complex is initiated. This change allows the virion to fuse with the target cell's membrane and results in virion internalization. From here the viral proteins spill into the cell and are ultimately incorporated into the cell's genome by way of a Reverse Transcriptase mediated pathway. Now known as a latent provirus, the viral genetic code requires a currently unknown impetus to initiate production of new viruses (Fields, 1998).

Endothelial dysfunction is a basic disturbance which can be associated with numerous pathologies of the cardiovascular system. It can involve small vessels and cause disorders such as vasculitis, or it can involve larger vessels and result in problems

as severe as aneurysms. Endothelial dysfunction can also plague coronary arteries and result in disorders of the heart itself affecting the pericardium, myocardium, or endocardium. One of the most clinically relevant consequences of endothelial dysfunction is the development of atherosclerotic plaques. These are believed to begin as activated patches of endothelium. This activation is characterized by endothelial expression of many proteins involved in monocyte attraction and adhesion such as ICAM, VCAM, IL-1, IL-8, and MCP-1. These activated monocytes become resident macrophages in the tissue and begin to ingest and accumulate cholesterol until they can hold no more. Now known as "foam cells," these macrophages begin to release cytokines such as TNFα that are toxic to the surrounding tissue. A fibrous cap is also formed which contains thrombogenic material at its core. Interestingly, the rate of formation of the fibrous cap is inhibited by IFN γ, a product of activated T cells. As this process continues the plaque becomes more severe with the replacement of normal elastic tissue with inelastic collagen, resulting in diminished contractility of the area (Libby, 2000).

Many articles exist relating HIV infection and disturbance in vascular function. Early reports associate small vessel lesions and an ocular microangiopathic syndrome with HIV infection (Pepose, Holland, Nestor, Cochran, 1985). Additionally, significant arterial pathology was shown in pediatric HIV cases (Joshi, Sharer, Pawel, Connor, Oleske, 1987). Endothelial leakage and elevated endothelial permeability have been reported by separate authors (Gariano, Rickman, Freeman, 1993; Rhodes 1991). Considering that disturbance in vascular function and endothelial activation is a

fundamental aspect of atherogenesis, these changes could also result in the pathologies observed with HIV in other studies, such as premature atherosclerosis (Tabib, Greenland, Mercier, Loire, Mornex, 1992), venous thrombosis, and atheroembolism (Capron, Kim, Laurien, Bruneval, Feissinger, 1992).

Regulatory mechanisms exist to maintain normal functioning of the endothelium. It has been shown that many HIV patients have these regulatory mechanisms disturbed. This can lead to an increase in concentrations of various markers in the serum, such as von Willebrand Factor (vWF), soluble Thrombomodulin (sTM), Angiotensin Converting Enzyme (ACE), E-selectin (ELAM-1), and endothelin (Schved et al., 1992; Seigneur et al., 1997; Zietz et al., 1996). For instance, when the endothelium is damaged it is known to shed vWF, a protein that plays an important role in the coagulation cascade. A positive correlation between disease progression and vWF was shown in HIV positive patients, though it was not proven conclusively that this was a direct result of HIV (Lafeuillade et al. 1992). This is believed to be a possible predisposing factor to thrombus formation. Other evidence also shows that endothelial damage is linked to HIV infection. sTM was shown to be negatively correlated with patients' CD4+ cell counts, which decrease as the HIV infection progresses (Seigneur et al.). In fact, this study shows that vWF increases correlate with increases in inflammatory cytokines such as Tumor Necrosis Factor α (TNFα) and Interferon γ (IFNγ), whereas sTM correlation with these cytokines was poor. This implies that sTM may be a more reliable indicator of endothelial damage and vWF may reflect overall inflammatory conditions (Seigneur et al.). One of the most poignant studies on endothelial reaction in HIV patients shows

numerous pathologies involved. Zietz et al. examined aortic endothelium from HIV patients and discovered a severely disturbed endothelial cell pattern when compared with normal patients. This study showed a significant increase in the number of leukocytes attached to the endothelium. This finding was bolstered by an increase in endothelial expression of Vascular Cell Adhesion Molecule (VCAM-1) and Endothelial Leukocyte Adhesion Molecule 1 (ELAM-1), which are necessary components in leukocyte adherence. Additionally, they found an increase in endothelial cell turnover rates.

Another group of proteins found in plasma are the soluble forms of Human Leukocyte Antigen (HLA) types I and II. These proteins circulate in plasma at low levels in healthy patients. HLA are involved in the presentation of non-self antigens to CD4+ and CD8+ T cells, binding to the T cell receptor (TCR) and either CD4 or CD8. HLA-I are found on most nucleated cells. Generally, professional Antigen Presenting Cells (APC) are the main population expressing HLA-II. Under more adverse conditions, such as inflammation, nonprofessional APC can also exhibit HLA-II proteins (Filaci et al., 1995). HLA-I is involved in presenting antigens derived from inside the cell. As a result, HLA-I is much more active during viral and intracellular infections. HLA-II is the protein that displays antigens which have been introduced into the cell through endocytosis. Therefore, HLA-II is more active during extracellular infections. Puppo et al. (1994) found that long term observation of HLA-I levels was a more accurate predictor of conversion to AIDS than is checking beta 2μ or neopterin levels, both previously believed to be strong indicators. Still, it was not as accurate as checking the patients' CD4+ cell count. This would seem logical as HIV patients have a primary

retroviral infection, and possibly subclinical secondary viral infections. Another study discovered an increase in HLA-II in HIV patients. In this study, HIV positive patients displayed an HLA-II value over twice as high as the controls. Those patients with full-blown AIDS, interestingly, showed HLA-II values approaching eight times the controls. This could affect immune response by binding CD4 or TCR on circulating T cells and blocking any further response from them (Filaci et al.).

Of profound importance to research studying this vasculopathy are two HIV proteins, tat and gp120. Typically, the tat (transactivator of transcription) protein is seen as a vital transcription factor that is only active after a virus has infected a cell. The normal role of gp120, on the other hand, is as a membrane bound protein that allows attachment of the virion to the CD4 marker on CD4+ T cells (Fields, 1998). These proteins are usually seen as integral to the viral life cycle, not as rogue elements which can alone act as cellular pathogens. However, HIV patients have been shown to have detectable levels of gp120 and tat circulating in their plasma (Ensoli et al., 1993; Oh et al., 1992). This shows us that the opportunity exists for these proteins to interact with uninfected cells. Naturally, one of the most predominant cell types to be highly exposed to any plasma protein is the vascular endothelial cell.

Endothelial cells typically regulate the passage of various substances between the blood and interstitium. They are also important in maintenance of normal coagulation and in the recruitment of leukocytes to inflamed areas of tissue. This makes endothelium highly important to the progression and outcome of various infectious and inflammatory diseases (Krishnaswamy et al. 1998; Krishnaswamy, Kelley, Yara, Smith, Chi, 1999).

Some types of endothelial cells have been shown to react to these circulating HIV proteins. One study examined the effects of tat (100ng/mL) with a six-hour treatment on the production of adhesion molecules in human umbilical vein endothelial cells. The study shows profound increases in expression of Intercellular Adhesion Molecule (ICAM-1), VCAM-1, and ELAM-1, all important molecules in the movement of leukocytes from the circulation to the tissue. Additionally, the study showed that the production of the adhesion molecules was completely blocked by addition of cycloheximide, indicating that they are products of *de novo* synthesis (Dhawan et al., 1997). A different study reported the effects of tat (10 ng/mL) treatment on central nervous system derived endothelial cells. The HIV protein was shown to increase IL-6 production by two to three fold over control values (Zidovetzki, Wang, Chin, Jeyaseelan, Hofman, 1998). Others also report tat dependent alterations in endothelial cytokine production such as increases in IL-2, IL-8, and Tumor Growth Factor β_1 (TGFβ_1) (Cupp, Taylor, Khalili, Amini, 1993; Hofman, Chen, Incardona, Zidovetzki, Hinton, 1999; Westendorp, Li-Weber, Frank, Krammer, 1994). Aberrant cytokine production could not only alter the immediate environment near the response, but could also influence the way other endothelial cells respond to tat. Fiorelli et al.(1999) showed that an increase in ambient IFNγ levels preceding tat treatment induced endothelial cells to respond by proliferating and invading the extracellular matrix (possibly helping to explain an HIV associated disease, Kaposi's sarcoma).

The Nuclear Factor κ B (NFκB) second messenger pathway is known to be involved in many inflammatory responses. The transcription factor, NFκB, is normally

bound by a cytoplasmic inhibitor protein, Inhibitor κ B (IκB). When this pathway is stimulated, IκB releases NFκB which then enters the nucleus and acts in regulation of pro-inflammatory genes. Though NFκB was known to be activated in the brains of HIV infected children, it was not known by what mechanism this was occurring (Dollard et al., 1995). The tat protein has since been shown to activate the NFκB pathway in astrocytes. Studying U373 astrocytes, Nath, Conant, Chen, Scott, Major (1999) showed a significant increase in activation of NFκB after incubation with 100 ng/mL of tat. They said this pathway was responsible for the release of IL-1 and IL-6 they were observing from the same cells. Because these same cytokines are often responsible for activating NFκB in the first place, it is probable that a positive feedback loop was being set up inducing a continuous amplification of cytokine production.

The Protein Kinase C PKC pathway is a second messenger system typically associated with numerous cellular responses, from stress reactions to growth stimulation. Activation of PKC has also been implicated in tat mediated cellular responses. Phorbol myristate acetate (PMA), an activator of the PKC pathway, was shown to synergize with tat in trans activating the HIV-1 Long Terminal Repeat (LTR) linked reporter gene (Laurence, Sikder, Jhaveri, Salmon, 1990). Also, PKC depletion was shown to degrade tat's ability to stimulate this same gene (Jakobovits, Rosenthal, Capon, 1990).

Though it appears tat is very important in endothelial alterations, gp120 also seems to have discernable effects. For example, gp120 treatment at a concentration of 0.1 μg/mL results in significant increases in monocyte adhesion to endothelium (Stefano, Salzet, Bilfinger, 1998). Interestingly, this report also showed that treatment of the

gp120 to endothelium does not cause the secretion of nitric oxide (NO), usually important in inflammatory processes. Looking at brain endothelium, Annunziata, Cioni, Toneatto, Paccagnini (1998) showed significant increases in endothelial albumin permeability when exposed to concentrations of gp120 as low as 10 pM. Again, the report highlights a lack of NO involvement in gp120 stimulation, as they showed no change in gp120 induced albumin permeability after blocking NO production with nitro-L-arginine methyl ester (L-NAME), a NO synthase inhibitor. Another report has implicated apoptosis in the normal endothelial cell response to gp120. Using human umbilical vein endothelial cells, the group exposed gp120 at various doses and discovered a biphasic response in cytotoxicity, peaking at both .01 ng/mL and 26 ng/mL. They followed this with morphology and DNA fragmentation studies and showed that apoptotic programs are being activated (Huang, Krishnaswamy, Su, Xiao, Liu, 1999).

The Mitogen Activated Protein Kinase (MAP Kinase) pathway is a highly branched second messenger pathway. There are numerous effects of stimulation of this pathway, from cell death to cell growth. The effect depends on which section of the cascade is activated. Stimulation occurred during gp120 exposure in both the JNK and ERK pathways of the MAP Kinase phosphorylation cascade in primary CNS cell cultures consisting of astrocytes, fibroblasts, and microglial cells. The JNK/p38 pathway is classically considered to be associated with apoptosis, while the ERK pathway is connected to cell growth and development. These authors hypothesized that their results show that a balance is necessary between activation of JNK/p38 and inhibition of ERK in order for apoptosis to occur (Lannuzel et al., 1997). A very similar result was achieved

by an earlier group studying the balance between stress and growth factor activated MAP Kinase pathways in pheochromocytoma cells (Xia, Dickens, Raingeaud, Davis, Greenberg, 1995). Considering this has now been shown in numerous cell lines, it is probable that this is a highly conserved response across human cell types.

Endothelial activation as a feature of HIV infection may lead to vascular disease. Endothelial cell activation in HIV infection may be due to the virus itself, inflammatory cytokines (IL-1β and TNFα), or secreted viral proteins gp120 and tat. This study has focused on these two HIV proteins as well as two cytokines, TNFα and IL-1β, and their effect on endothelial cell activation. We assessed endothelial cell signaling via the MAP Kinase and the NFκB pathways, both critical pathways for gene expression. We also evaluated endothelial cell production of two inflammatory cytokines, IL-6 and IL-8, capable of inducing the acute phase response, cellular chemotaxis, and leukocyte recruitment- all features of vascular disease.

CHAPTER 2

METHODS AND MATERIALS

Cell Culture

Human Pulmonary Artery Endothelial Cells (HPAEC) from BioWhittaker/Clonetics (Walkersville, MD) were removed from liquid nitrogen storage and cultured in 75 cm^2 tissue culture flasks (Corning). The growth medium was also purchased from BioWhittaker/Clonetics as a kit consisting of Endothelial Base Media, 10 ng/mL Endothelial Growth Factor, 1 mg/mL Hydrocortisone, 50 mg/mL Gentamicin, 50 µg/mL Amphotericin B, 3 mg/mL Bovine Brain Extract, and 10% Fetal Bovine Serum. The medium was changed every 3-4 days. HPAEC were grown in an incubator with a steady atmosphere of 5% CO_2 at 37° C. Once the cells were near confluency, they were removed from the flask by trypsinization and split to two new 75 cm^2 flasks.

Trypan Blue Exclusion

When the HPAEC were to be plated for an experiment, the cells were removed by trypsinization and centrifuged at 1000g for 10 minutes for concentration. These cells were resuspended in media, a small volume was removed, and Trypan Blue was added. The cells were then viewed on a hemocytometer. This procedure allowed the cell concentration and viability to be determined. The appropriate number of cells could then be transferred to the experimental well. Cells were not used if viability was below 80%.

HIV Protein Treatment

HPAEC were trypsinized and counted, then 200,000 cells were added to each well in 12 well culture plates from Becton-Dickinson (Lincoln Park, New Jersey). These cells were allowed to attach and grow overnight. The cells were then incubated for 24 hours with varying doses of tat and gp120, or with combinations of other proteins of interest such as IL-1 or TNFα. After this treatment, the supernatants were removed and subjected to a short centrifugation to remove particulates. Supernatants from this type of experiment could then be frozen at -20° C until used for further testing.

Enzyme Linked Immunosorbent Assay (ELISA)

Supernatants were removed after the cells had completed a 24-hour treatment to the agents of interest. Samples were assayed immediately or stored at -20° C. The ELISA kits being used for this assay were purchased from R&D Systems (Minneapolis, MN), where an antibody specific for a particular cytokine/ chemokine was bound to each well in the plate. These primary antibodies have been shown to react solely with the protein of interest. The assays were based on methods described by Voller, Bidwell, Bartlett (1976).

Following manufacturer's instructions, each serial dilution was pipetted into duplicate wells. The standard acted as a series of specific concentrations of the cytokine/ chemokine of interest. The supernatants to be tested were then pipetted into the remaining wells. This entire series of wells was then incubated for two hours at room temperature to allow binding between the cytokine/ chemokine of interest and the

antibody attached to the well.

After the 2-hour incubation, the wells were emptied of their contents. The wash buffer was added to each well, and was then rinsed out. This process was repeated five times, resulting in the removal of all unbound material. All that remained was the protein that was bound to the antibody coated on the well.

When the wash was complete, a second solution was added to the wells consisting of a secondary antibody. The secondary antibody had an oxidative enzyme attached to the F_c portion. This protein binds its variable region to the cytokine or chemokine which was bound to the primary antibody, resulting in a 1:1 ratio of protein to oxidative enzyme. Again, a series of five washes was used to remove all unbound secondary antibody.

At that time, the substrate solution was added. It reacts with the enzyme on the secondary antibody to create a colored product. Because these react for a set amount of time at the same temperatures, the kinetics of the reactions are identical. The only variable that existed was the amount of enzyme present, and therefore the amount of the cytokine or chemokine of interest. The extent of color change was directly proportional to the amount of the cytokine or chemokine.

The colored products were measured on a Dynatech MR5000 spectrophotometer at a wavelength specific for that colored product (450 nm). Data imported from this were examined using BioLinx 2.22 software. By reading the products in the serial dilution wells, a standard curve could be developed from which the unknown sample concentrations were determined.

Determination of Protein Concentration

The protein concentrations in the solutions were determined by a commercial BCA assay (Pierce Chemical Co., Rockford, IL)(Stoscheck, 1990). BCA solution added to the buffer (10% SDS solution for Western Blot, Buffer C for NFκB procedure) was used as a zero standard, and solutions of known protein concentrations were used as appropriate additional standards.

Western Blot

This procedure is modified from the earlier works of Harlow (1988) and Bollag (1996). After a 45-minute treatment the HPAEC were rinsed with 5 mL of cold PBS. The cytoplasmic proteins were isolated from cells by direct treatment with cold Triton X and digitonin solution to lyse the cell membranes. This mixture was allowed to sit on ice before cells are scraped off the bottom using a rubber cell scraper. This cell lysate and Triton X solution was then stored at -20° C until the blot was run.

After the protein concentrations were determined using the BCA method, equal amounts of protein were added to each well on an 8% acrylamide gel for electrophoresis. After the proteins have been separated by this method, the gel was placed against nitrocellulose paper and the bands transferred to the paper by the application of current. The blot, as the paper is now referred to, has nonspecific binding sites blocked by addition of blocking buffer, and was then incubated with a primary antibody. This was followed by the addition of a secondary antibody. A chemiluminescent substrate was added, and the blot was then exposed to film for 15-30 minutes.

Nuclear Factor Kappa B Isolation and Measurement

The gel shift assay performed for NFκB measurement was based on published methods (Li, Browder, Kao, 1999; Li et al., 2001). The HPAEC were exposed for two hours to the proteins under consideration. The supernatant was removed and the cells were scraped into 10 mL of cold PBS. After centrifugation, the cell pellet was resuspended in fresh PBS, moved to a microfuge tube, and pelleted again. The pellet was left on ice for 15 minutes after which 25 μL of 10% Nonidet NP-40 was added. This solution was vortexed and centrifuged, and to the resulting pellet was added 50 μL of high salt buffer (Buffer C). After this solution was shaken for at least 15 minutes at 4° C, the mixture was centrifuged. The final supernatant contained the nuclear proteins.

After determining the protein concentration by BCA assay, the protein was combined with a radioactive oligonucleotide. This oligonucleotide was constructed by combining [γ-^{32}P] ATP, an NFκB oligonucleotide, and a polynucleotide kinase. This reaction resulted in a ^{32}P labeled oligonucleotide product. The NFκB and the oligonucleotide were incubated together for 20 minutes, and the reaction was halted by the addition of gel loading buffer.

A 4% acrylamide gel was constructed for the separation of the NFκB subunits. After the gel was run, it was dried on a gel drier with nitrocellulose paper pressed against it. X-ray film was then exposed to this paper for 10-15 hours.

RNA Isolation

RNA extraction and isolation was performed by an accepted procedure

(Chomczynski, Sacchi, 1987). After a 6-hour treatment with HPAECs, the cells were rinsed with cold PBS and the cells are put on ice. One mL of RNAzole was added to each well containing cells. This was pipetted to remove and break up cells, as well as to digest the DNA, and then the solution was mixed with 0.1mL cold chloroform. This was centrifuged, and the top layer was removed. To this layer was added cold phenol-chloroform. After vortexing this mixture was centrifuged. The top layer was kept and cold chloroform was added, again followed by vortexing and centrifugation. The previous step was repeated. The top layer was retained and cold n-propanol was added. This mixture was then frozen overnight. On the second day this mixture was thawed and centrifuged. The RNA pellets at the bottom, and the remaining liquid was removed. The RNA was then washed with 0.1% DEPC and was then allowed to dry at room temperature. RNA was then resuspended in 20 µL 0.1% DEPC H_20. The concentration of the RNA was determined by using a spectrophotometer set at 260nm and 280nm. The 260/280 nm ratio determined the quality of the RNA. The 260 nm reading alone could be used to calculate the concentration of RNA (260x20,000 = X ng/µL RNA). The RNA was then stored at -80° C.

Reverse Transcriptase Polymerase Chain Reaction

Initially, RNA was isolated, quantified, and stored as described above. 200 ng RNA could then be combined with tubes of Reverse Transcriptase mix (Buffer, $MgCL_2$, dGTP, dATP, dCTP, dTTP, Oligonucleotide, RNAse Inhibitor, Reverse Transcriptase, H_2O) and run on a thermocycler (Perkin-Elmer DNA Thermocycler 480) at 42° C for 20

minutes, 99° C for 10 minutes, and 5° C for 5 minutes. This procedure yields cDNA translated from the original RNA.

This DNA was then combined in a new tube with MgCl2, Buffer, H2O, and TAQ Polymerase. Then to the individual tubes were added the 3' and 5' primers for the genes of interest. Mineral oil was placed on top of this mixture to keep all of the aqueous components together in the tube and prevent different rates of reaction. This solution was then run on the thermocycler for 45 cycles (though this number can be altered depending on the gene being studied), each cycle consisting of 95° C for 45 seconds, 60° C for 45 seconds, and 72° C for 90 seconds.

The product of this RTPCR was then put in the lanes of a 3% agarose gel and run at no more than 80 V until completion. The banding pattern of the DNA lines up with the molecular weight markers consistent to the gene being amplified. Differences in expression are seen by variations in band density (Huang, Krishnaswamy, Su, Xiao, Liu, 1994; Krishnaswamy et al., 1993).

HIV Proteins Tat and Gp120

The recombinant HIV-1 tat protein was produced in E. coli and was obtained through the AIDS Research and Reagent Program, NIAID, NIH. from Dr. John Brady (Bohan et al., 1992; Gutekunst, Kashanchi, Brady, Bednarik, 1993; Kashanchi, Duvall, Brady, 1992; Kashanchi, et al. 1994; Kashanchi, Shibata, Ross, Brady, Martin, 1994).

The recombinant HIV-1 LAV gp120 was produced in the Baculovirus Expression System and obtained from Immuno Diagnostics, Inc (Woburn, MA).

CHAPTER 3

RESULTS

Cytokine Expression

This series of experiments was designed to highlight the development of particular cytokines upon HPAEC exposure to tat or gp120. The concentrations of tat and gp120 were chosen based on results from other studies using endothelial cell lines (Annunziata et al., 1998; Dhawan et al., 1997; Hofman et al. 1999; Huang, Hunter, Bond, 1999; Zidovetzki, et al. 1998). After the 24-hour incubation cell supernatants were obtained and tested by ELISA for IL-6 and IL-8 concentrations (Figure 1). Incubation of HPAEC with tat (10, 100, and 500 ng/mL) showed no significant difference in IL-6 production compared with the control IL-6 values. However, IL-6 secretion was significantly increased by tat at 250 ng/mL (p=.02). Similarly, incubation of HPAEC with gp120 showed no significant increases in the production of IL-6 over control. Treatment with both gp120 (250 ng/mL) and tat (100 ng/mL) showed no synergistic activation of IL-6.

The tat incubations at levels of 10 ng/mL and 100 ng/mL showed no significant increase over the control for secretion of IL-8 (Figure 1). After treatment to tat at 250 ng/mL, however, the cells showed a dramatic increase (p=.03) in IL-8 secretion. Also, a 500 ng/mL dose of tat resulted in significant depression in IL-8 values against the control (p=.02). The incubations with gp120 doses showed no significant differences in secretion of IL-8 when compared with the control. The combination of gp120 (250

ng/mL) and tat (100 ng/mL) also showed no significant increase in IL-8 secretion compared with baseline.

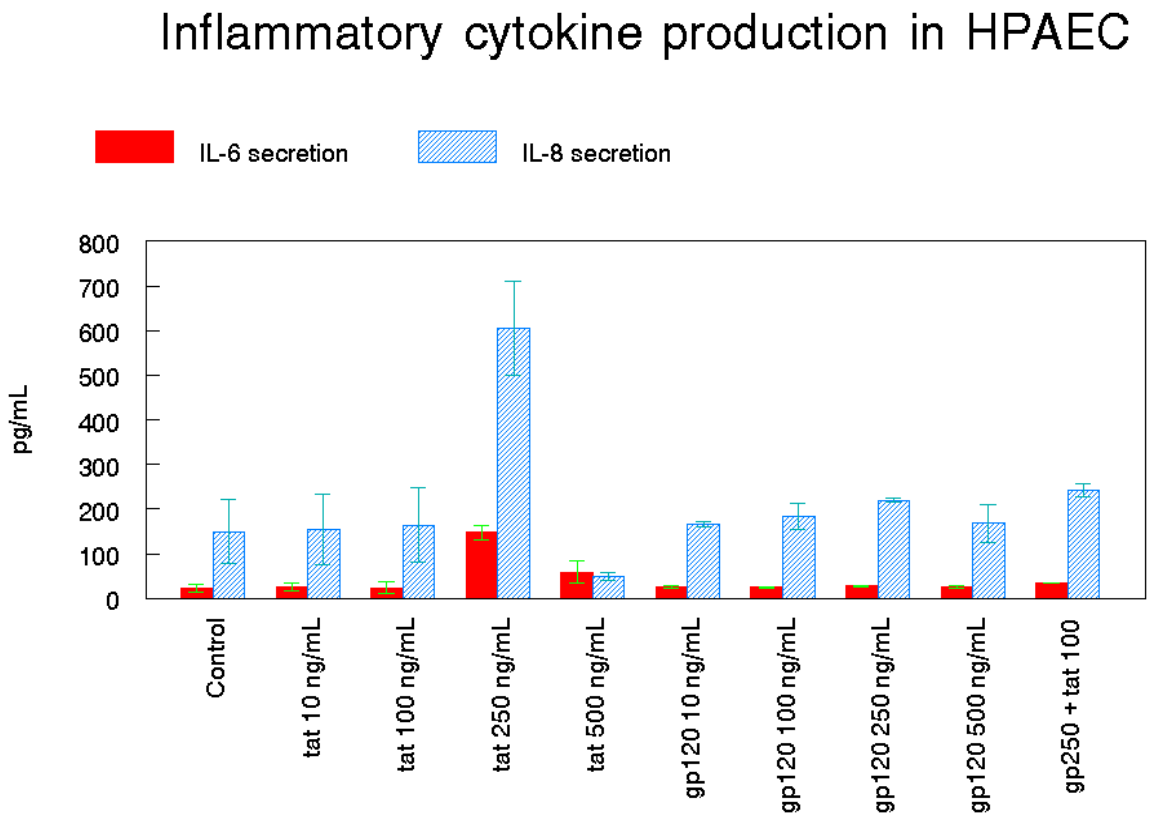


Figure 1: This figure shows the IL-6 and IL-8 supernatant levels after a 24-hour treatment with the HIV proteins at the concentrations indicated. The experiment was performed in triplicate. Only tat (250 ng/mL) resulted in a significant increase in production of both IL-6 (p=.02) and IL-8 (p=.03), and only tat (500 ng/mL) significantly depressed the production of IL-8 (p=.02).

IL-8 secretion was also tested after a treatment with tat, gp120, TNFα, and IL-1. This experiment showed very little difference in secretion of IL-8 after stimulation with TNFα or IL-1 treatment, with or without tat and gp120 (Figure 2). When MIP1a was assayed, however, a more interesting result was seen. The IL-1 activated the cells to secrete MIP1a, but the TNFα and HIV proteins did not change drastically from the baseline (Figure 3). When the IL-1 was combined with the tat and gp120, most of the responses were similar to the IL-1 control. But when tat (500 ng/mL) was added to the IL-1, the MIP1a secretion was drastically reduced to levels very similar to the baseline. Also, the TNFα added to the HIV proteins had no significant effects except a decrease in MIP1a secretion with gp120 (500ng/mL). MCP1 secretion by the HPAEC's was also assayed in the same manner. No significant differences were seen in MCP1 secretion as any of the proteins were added (Figure 4).

The secretion of IL-6 is shown in Figure 5. Though the gp120 treatments show little difference in IL-6 secretion, the tat treatments show a significant increase at 250 ng/mL and even more profound increase at 500 ng/mL. The IL-1 treatments result in IL-6 secretion that is very similar to the control. The gp120 treatments also show very little change from the control. The TNF and gp120 treatments show almost no alterations from their baseline, but the TNF and tat treatments show a significant departure from this trend. At 10 and 100 ng/mL, the IL-6 production changes very little. But at 250 ng/mL the IL-6 secretion begins to rise, and at 500 ng/mL it shows an even higher value.

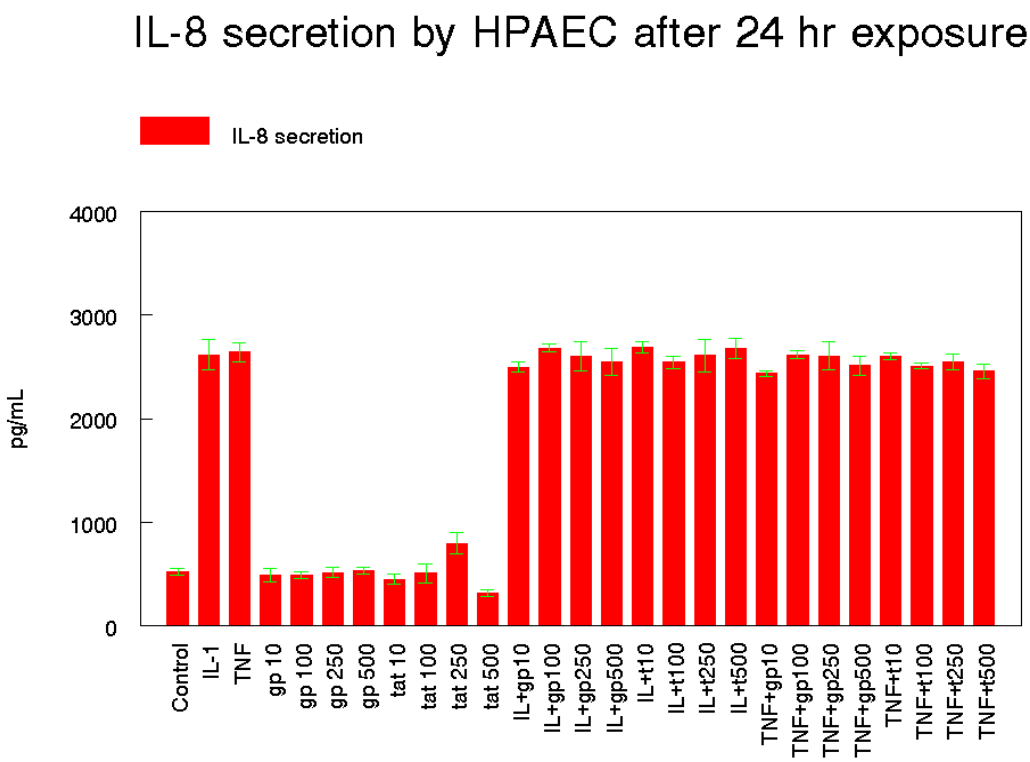


Figure 2: Triplicate stimulation of HPAEC with different combinations of gp120 (shown as gp10 for gp120 (10 ng/mL), tat (shown as t10 for tat (10 ng/mL), TNF (100 units/mL), and IL-1 (10ng/mL). The tat protein alone showed some interesting effects on IL-8 production (see Figure 1), but neither gp120, TNF, or IL-1 caused any alteration in IL-8 production compared to their respective control values.

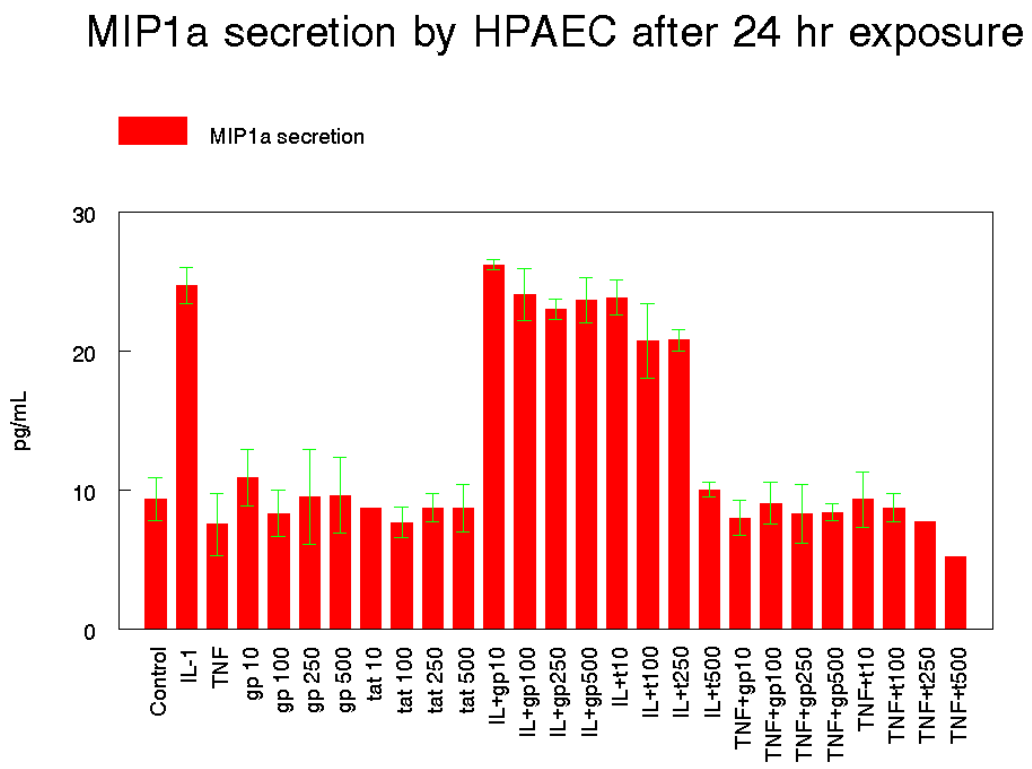


Figure 3: ELISA results of supernatant concentrations of MIP1a after a triplicate 24-hour treatment to gp120 (listed as gp10 for gp120 (10ng/mL), gp100 for gp120 (100 ng/mL), etc.), tat (listed as t10 for tat (10 ng/mL), t100 for tat (100 ng/mL), etc), IL-1 (10ng/mL), and TNFα (100 units/mL). The most significant changes result with IL-1 + tat (500 ng/mL). This costimulation returned MIP1a secretion to control values. Also, TNFα + tat (500 ng/mL) caused a decrease in secretion of MIP1a below the control concentration.

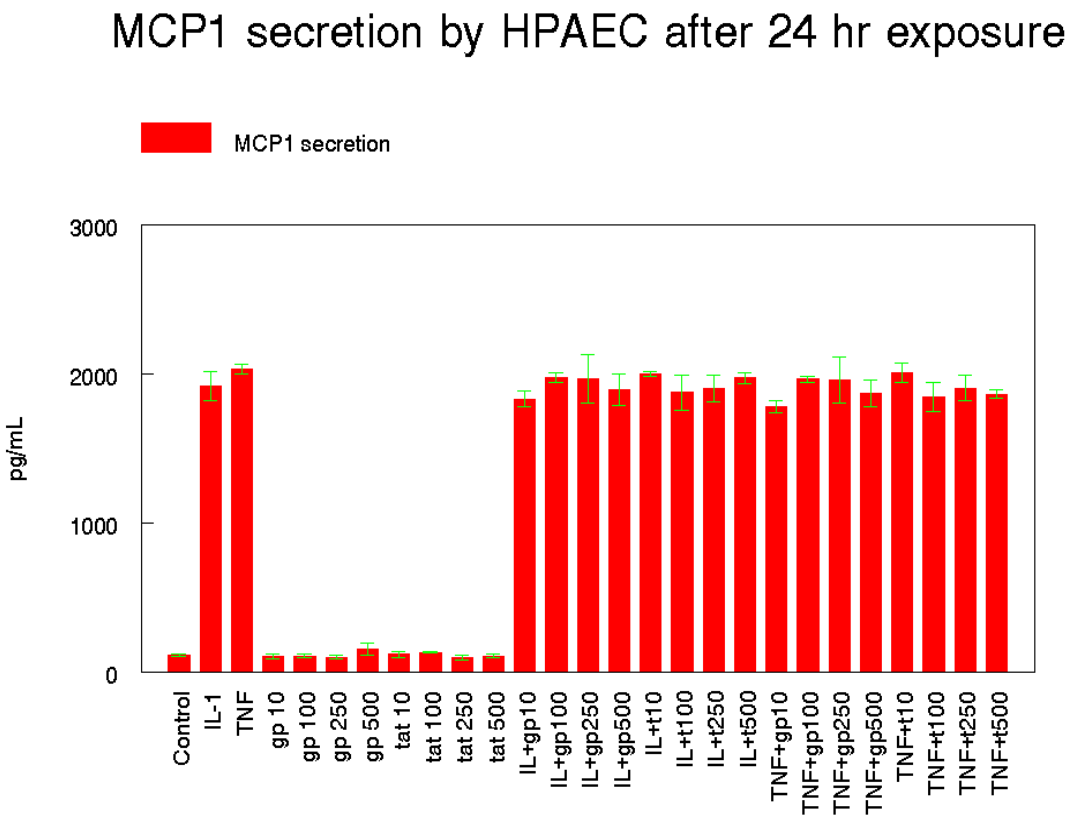


Figure 4: MCP1 concentrations after 24-hour treatment to proteins. No combination of protein caused any significant change from baseline in MCP1 secretion.

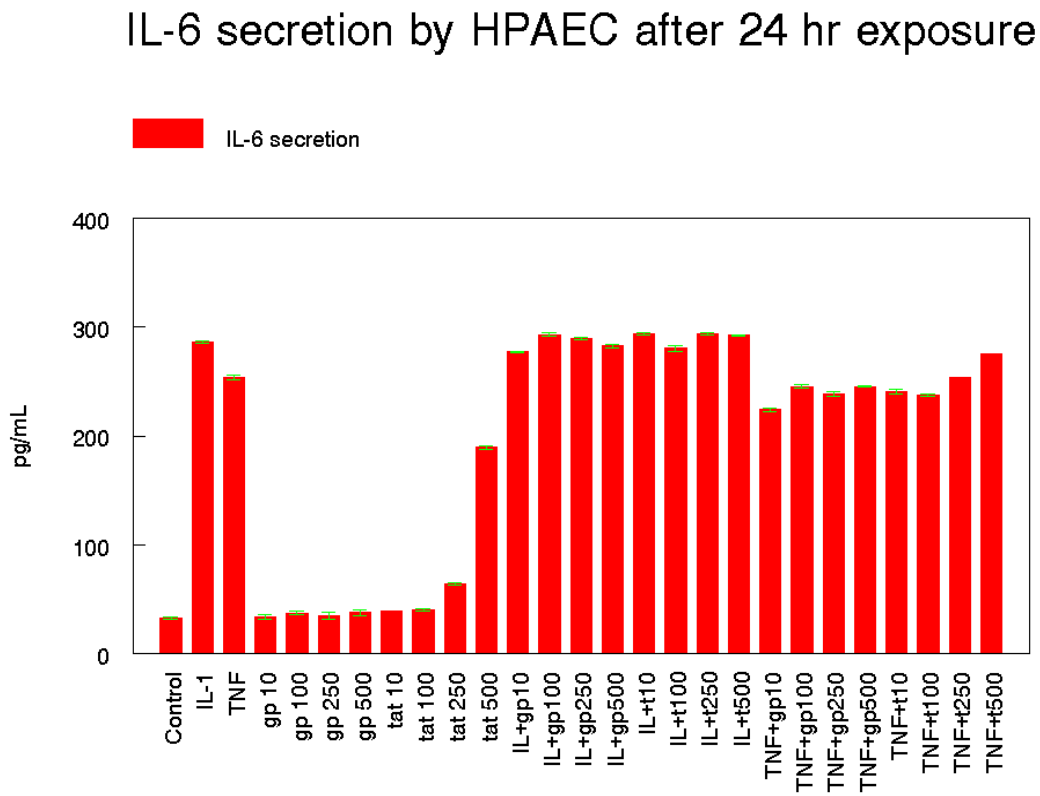


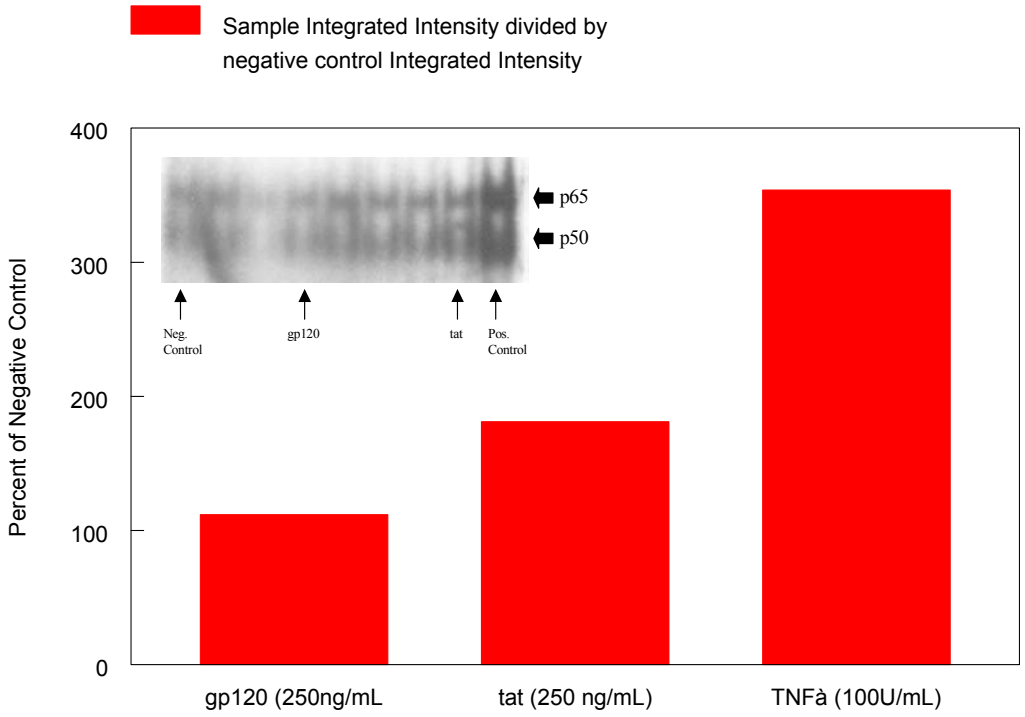
Figure 5: IL-6 secretion by HPAEC. An increase is seen in treatment with tat (250 ng/mL) followed by a large increase with tat (500 ng/mL). Also, treatment with both TNFα (100 U/mL) and tat (500 ng/mL) shows a synergistic increase in IL-6 secretion when compared to other TNFα treatments.

Second Messenger Pathway Involvement

The experiments detailing NFκB activity were designed to show the loss of inhibition and, subsequently, the translocation to the nucleus of active NFκB. Figure 6 shows the involvement of NFκB in the stimulation of HPAEC. Because nuclear proteins were isolated for this assay, the results reflect only protein that has translocated to the nucleus. Briefly, the cells were grown to near confluency in a T-75 flask and exposed to the proteins of interest for two hours. This time was chosen because earlier experimentation showed a marked decrease in NFκB activity after four hours and a peak at two hours (Figure 7). After the two hour incubation the cells were rinsed with cold PBS and scraped using a cell scraper. The nuclear proteins were then isolated from the resulting cell suspension. These cells show only a 12% increase over control in phosphorylation of NFκB after treatment to 250 ng/mL of gp120. A 250 ng/mL dose of tat, however, increases NFκB phosphorylation by 81%. Because the p65/p50 heterodimer of NFκB shows a distinct band and the p50/p50 homodimer does not, only the p65/p50 heterodimer band was measured.

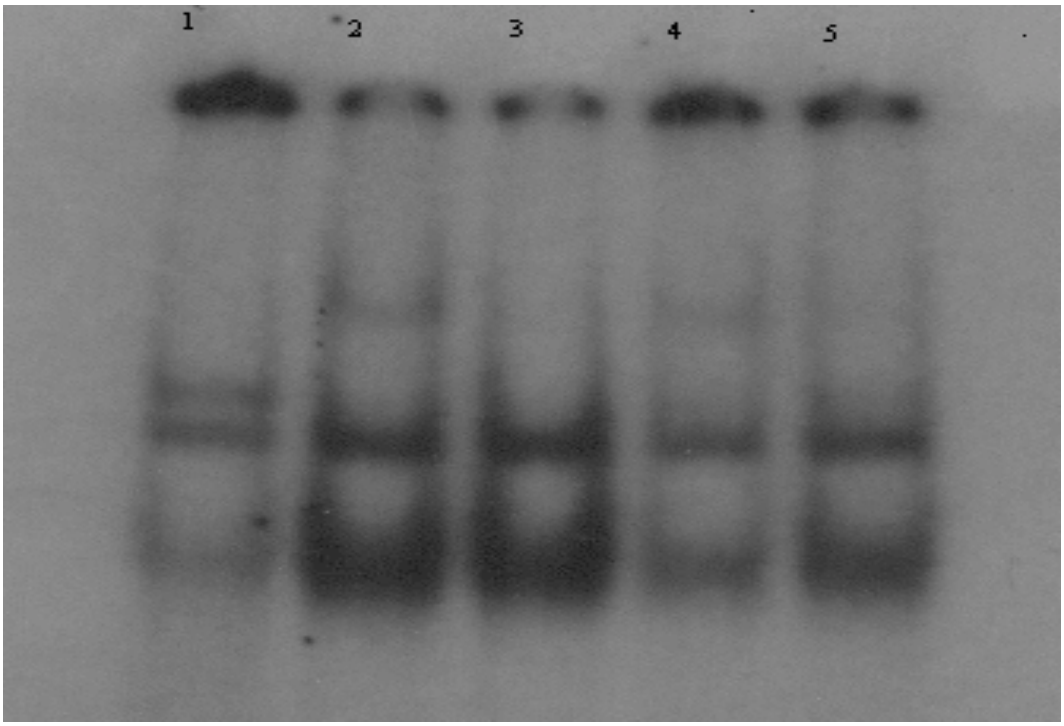
Considering the importance of other second messenger pathways in cell signaling, the involvement of the MAP kinase pathway was also assayed. The experiments studying MAP Kinase were made to determine time points and then determine rates and amounts of phosphorylation of key MAP Kinase protein intermediates. First, the amount of time to reach peak phosphorylation of the MAP kinase proteins was determined. This was achieved through activation of the cells with cytokines known to induce MAP kinase activity, TNFα and IL-1β (Xia, et al. 1995). HPAEC were exposed to TNFα and IL-1β at

four different time points (15, 30, 45, and 60 mins.). The cytoplasmic proteins were then



NFkB Activation in HPAEC

Figure 6: Phosphorylation of NFkB shown as a percentage of negative control. The gp120 (250 ng/mL) 2-hour treatment resulted in only a 12% increase in phosphorylation. The tat (250 ng/mL) 2-hour treatment caused an 81% increase in NFkB phosphorylation. The TNFα is a positive control.



NFkB Gel Shift Assay

Figure 7: Gel shift assay showing NFkB translocation in control (lane #1), TNF (100 U/mL) at two hours (lane #2), tat (250 ng/mL) at two hours (lane #3), TNF (100 U/mL) at four hours (lane #4), and tat (250 ng/mL) at four hours (lane #5). Equal amounts of protein were loaded to each lane.

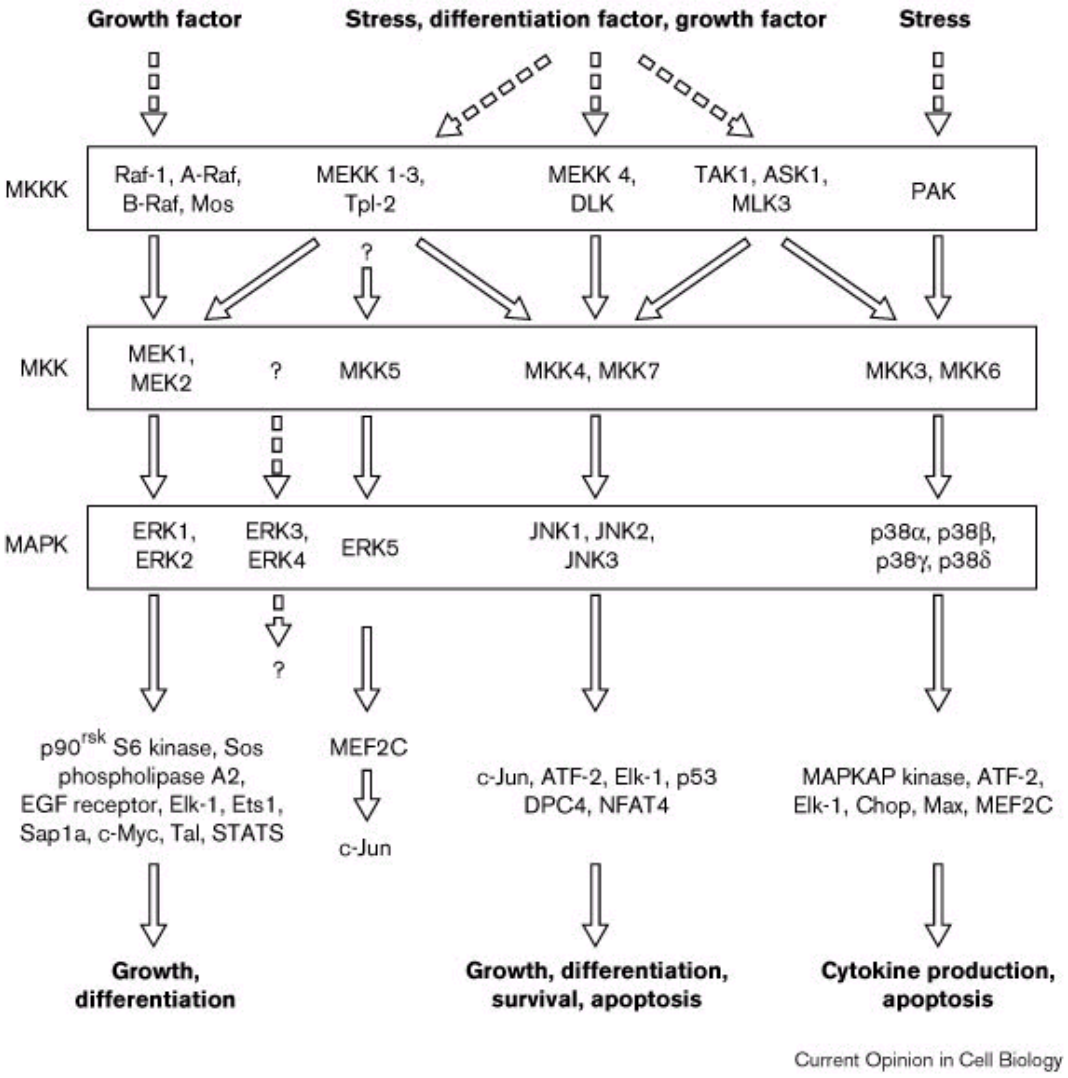
isolated and assayed by western blot for p38 phosphorylation, a late MAP kinase cascade protein known to be involved in cell stress responses and apoptosis (Figure 8) (Lannuzel et al., 1997; Xia et al., 1995). The 15 minute treatment showed an absence of phosphorylation (Figure 9). Thirty minutes showed an increase, and 45 minutes showed a very strong band. By 60 minutes the phosphorylation had started to diminish. This shows that the HPAEC MAP kinase p38 pathway is maximally stimulated at 45 minutes. The assay was then repeated for treatment with tat at 250 ng/mL, the only concentration with significant effects on cytokine production and NFκB activity.

Figure 10 displays the band intensity for post treatment p38 phosphorylation. The graph shows the control value followed by the tat, IL-1, TNF, and IL-1 + TNF treatments. The lowest point on the histogram is the control. The highest points are the combinations of IL-1 and TNF stimulations. Intermediate between the two is the phosphorylation induced by tat. This could indicate the tendency of tat to induce cells toward a stress-like response.

ERK1,2 were also examined by Western blot. Little difference was seen between the control and the tat 250 ng/mL (Figure 11). Though some increase is seen in the third lane (IL-1 10 ng/mL), the greatest increase in ERK1,2 phosphorylation is with TNFα 100 U/mL. When TNFα and IL-1 are used together no synergy occurs.

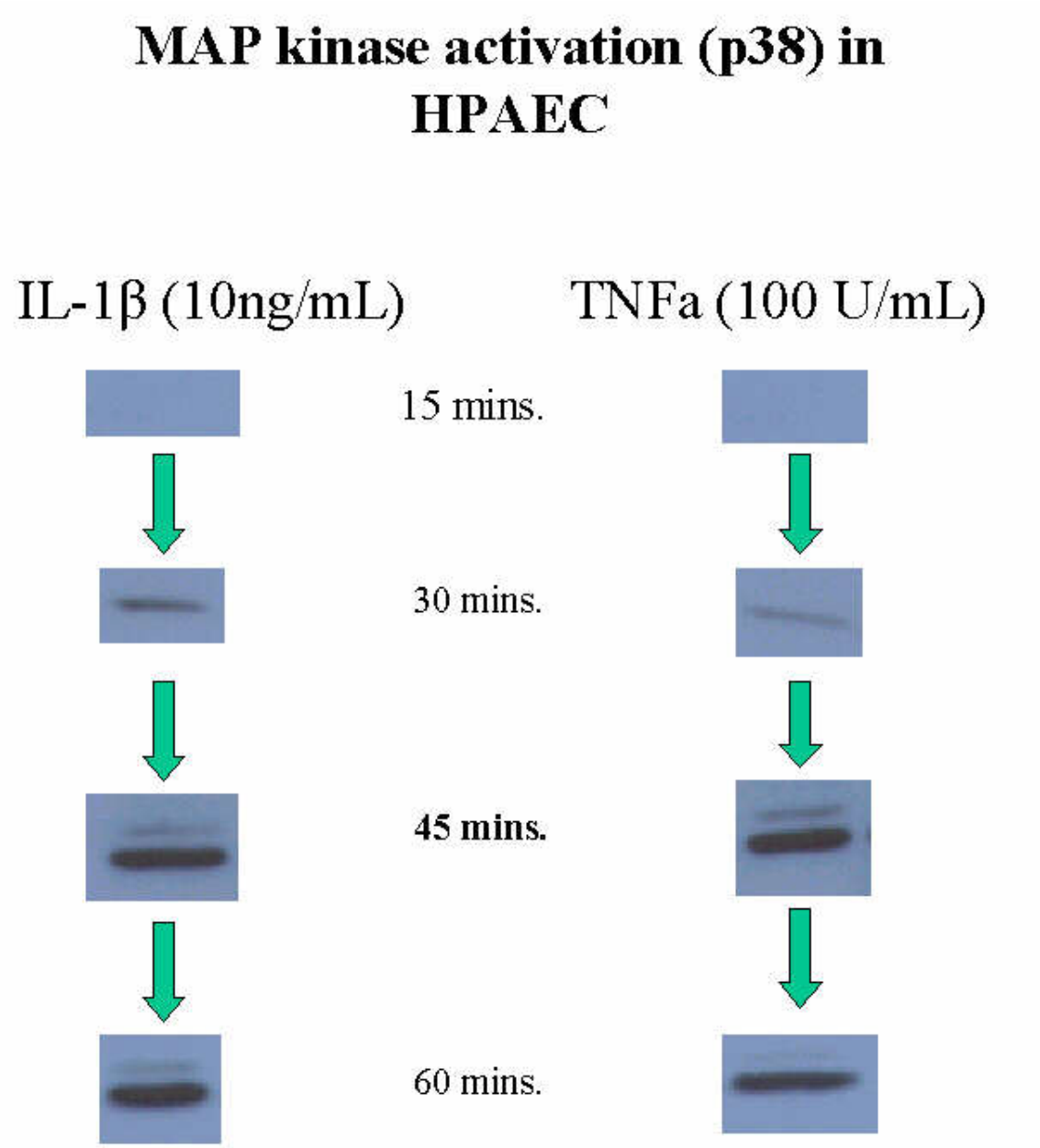
Morphology

Figure 12 shows the effects of HIV protein treatment on HPAEC morphology. Changes in cellular morphology can *in vitro* can be indicative of *in vivo* alterations, and



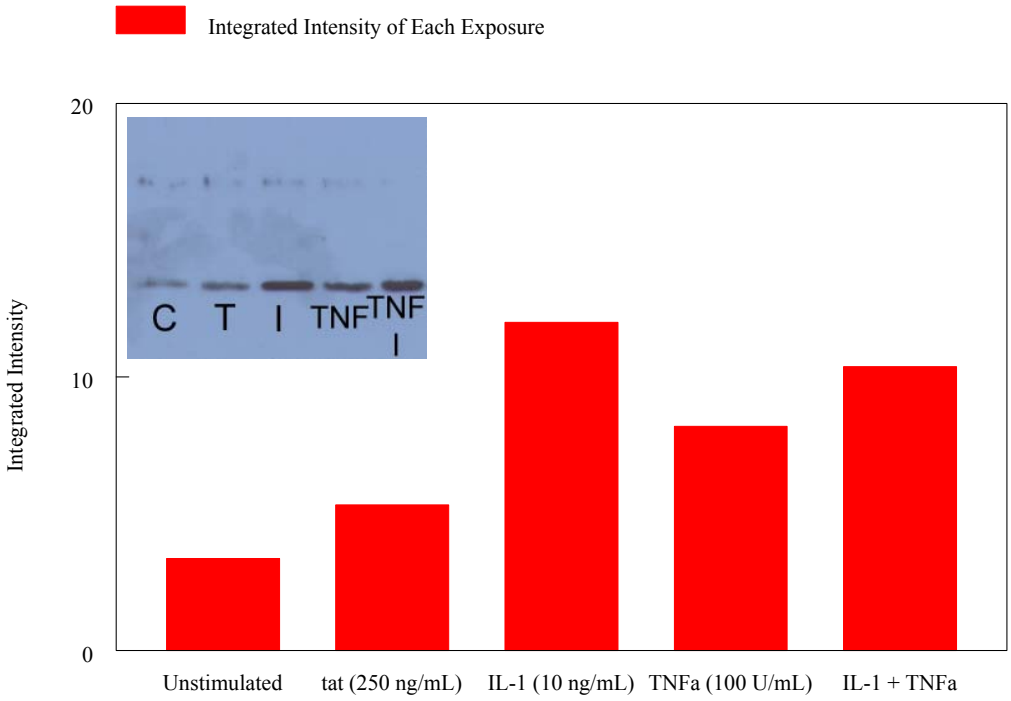
MAP Kinase Cascade Diagram

Figure 8: The interactions between many of the known MAP kinase cascade proteins. Both ERK1,2 and p38 are shown in the MAPK box. (Garrington, Johnson, 1999)



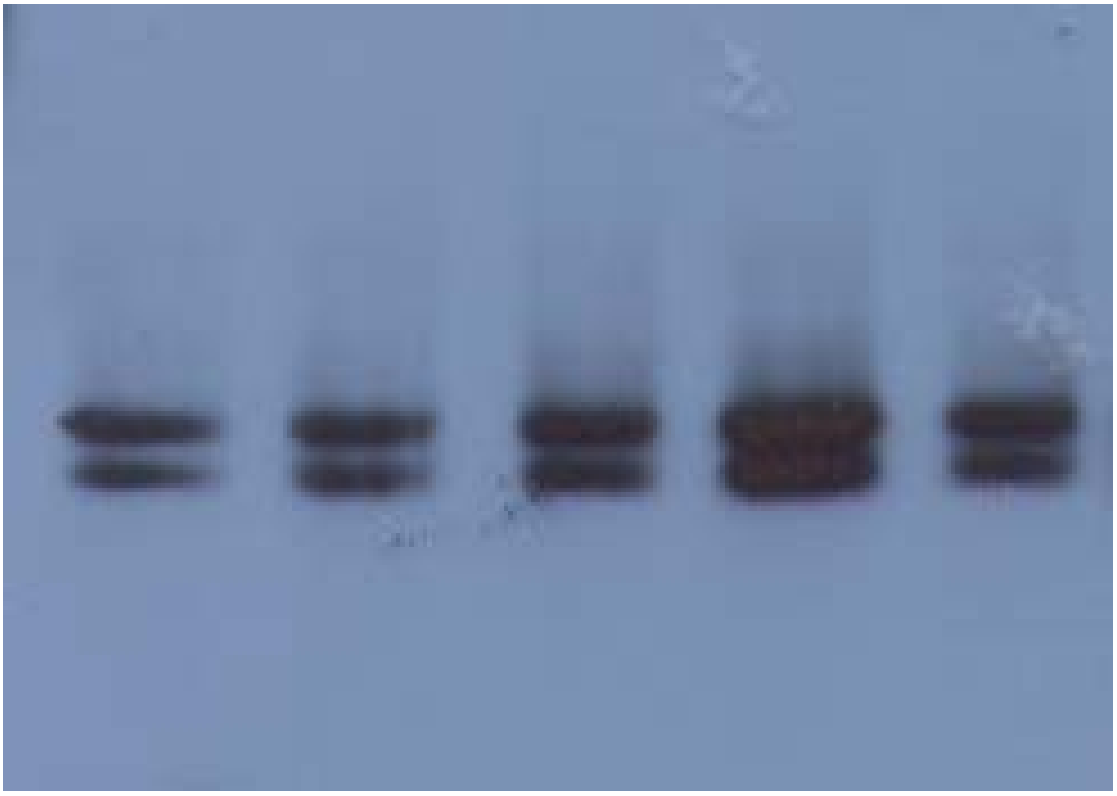
MAP Kinase Activation (P38) In HPAEC

Figure 9: This figure shows the time course of phosphorylation of p38 after treatment to IL-1 and TNFα. It increases until it peaks at 45 minutes and then begins to drop off by 60 minutes.



Phosphorylation of P38 By Western Blot

Figure 10: The band intensities of the Western Blot of proteins isolated after various treatments. The blot used a phospho-p38 specific antibody against cytoplasmic fractions of endothelial cells. Equal amounts of protein were loaded to each lane.



ERK1,2 Phosphorylation By Western Blot

Figure 11: A Western blot showing HPAEC ERK1,2 phosphorylation after a 45-minute incubation with control (left), tat 250 ng/mL (second lane), IL-1 10 ng/mL (middle), TNFα 100 U/mL (fourth lane), TNFα 100 U/mL + IL-1 10 ng/mL (right lane). Equal amounts of protein were loaded to each lane.

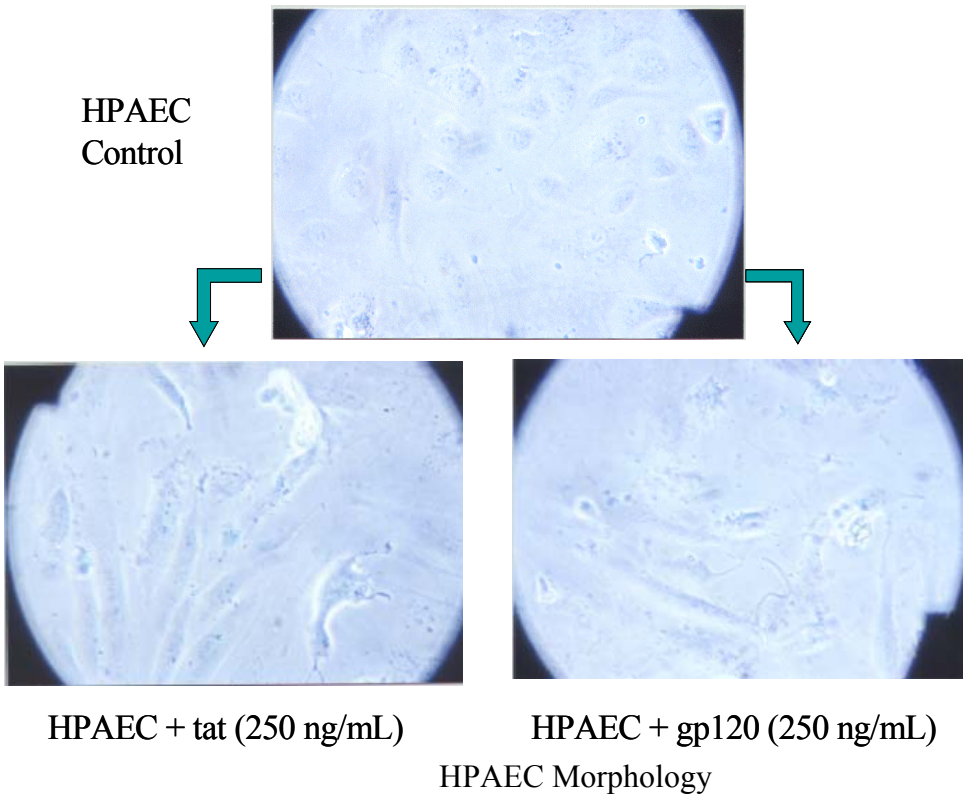


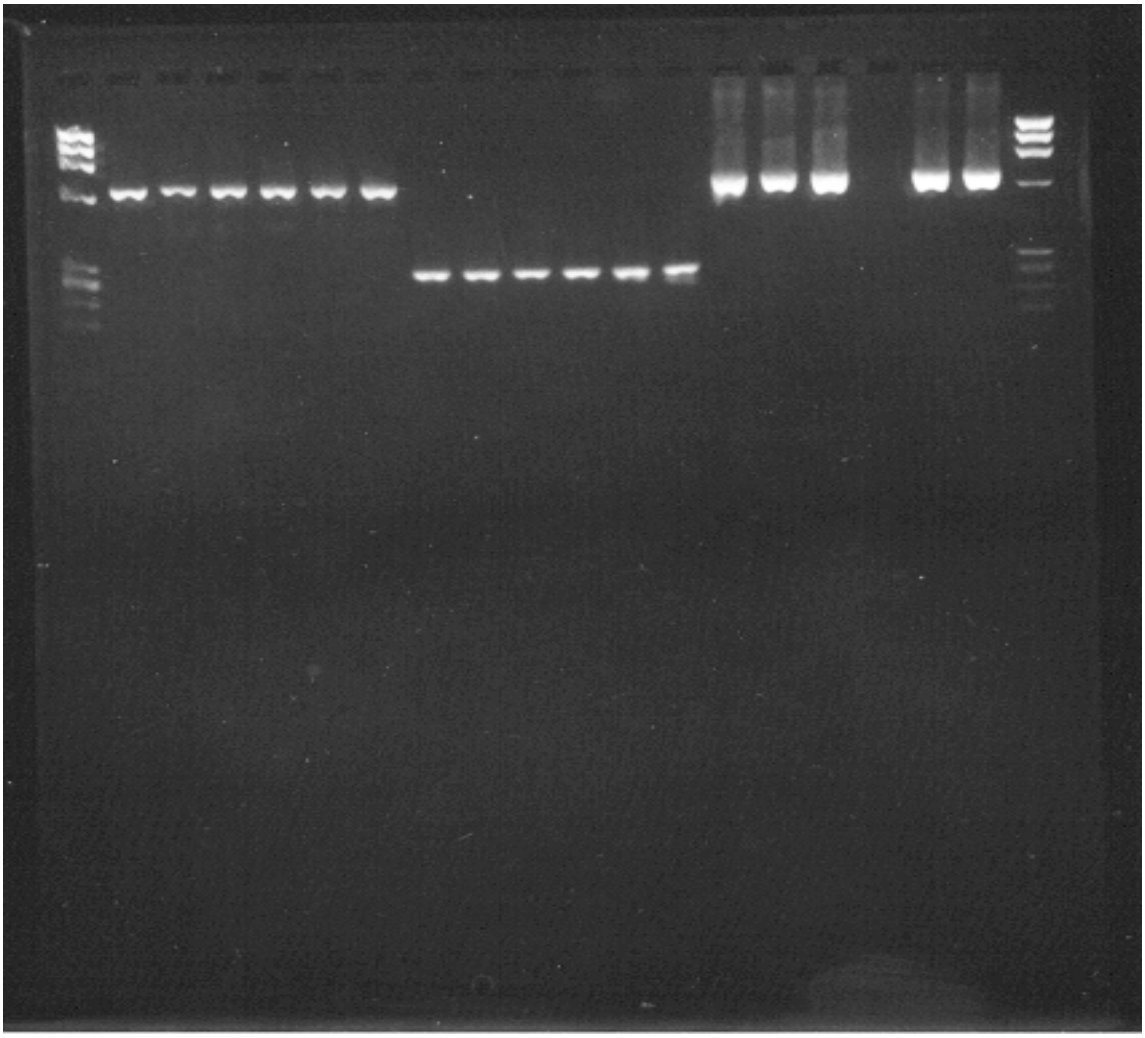
Figure 12: The uppermost picture shows the control morphology. Additional pictures were taken showing morphology after a 24-hour treatment to tat (250 ng/mL) and gp120 (250 ng/mL). These later pictures show an increase in vesicle formation and areas of cellular detachment.

these experiments would show if changes were occurring. The control shows normal morphology of attached HPAEC. These cells show significant physical alterations when exposed to tat at 250 ng/mL. A similar phenomenon is seen when the cells are exposed to a 250 ng/mL dose of gp120. In both treatments the cells become elongated and show signs of cytoskeletal rearrangement. These changes could have considerable importance to vascular pathology.

RNA Expression

After the RNA of a cell was harvested, it underwent RT-PCR to determine its corresponding DNA sequence. Exponential copying of these DNA sequences can then show the presences or absence of the RNA in this experiment for particular cytokines. The RNA of the exposed cells was harvested and RT-PCR was performed on it, both as described in Materials and Methods. The primers for IL-6, IL-8, and β-actin were used in this procedure to determine if any RNA was being produced for these proteins. The molecular weight markers are shown on both sides of the gel (Figure 13), and there are three groups of six bands each between the ladders. The group on the left is IL-6, the group in the middle is IL-8, and the group on the right is β-actin. The gel shows that all of these treatments seem to induce similar production of RNA for all of these proteins. The single exception on this gel is the fourth band for β-actin, which is not present. The lack of a β-actin band is probably a result of the primer not annealing correctly. β-actin has a variable sequence, and the primer used most likely had a sequence which was only complementary enough to usually work. This would explain the presence of other bands,

and yet the lack of this particular β-actin band.



Expression of Selected Proteins and Inflammatory Genes

Figure 13: The gel shows the results of RT-PCR on DNA isolated from endothelial cells. Ladders are on both edges, MW: 1353, 1078, 603, 310, 234, 194; the gel is stained with Ethidium Bromide. Each group has RNA from six experiments (left to right: Unstimulated, tat 100 ng/mL, tat 250 ng/mL, IL-1 10 ng/mL, TNF 100 U/mL, TNF + IL-1). The group on the left used a primer for IL-6, the group in the middle IL-8, and the group on the right β-actin.

CHAPTER 4

DISCUSSION

There are numerous effects of stimulation of the MAP Kinase pathway, from cell death to cell growth. The effect depends on which section of the cascade is activated. Stimulation occurred during gp120 exposure in both the JNK and ERK pathways of the MAP Kinase phosphorylation cascade in primary CNS cell cultures consisting of astrocytes, fibroblasts, and microglial cells. The JNK/p38 pathway is classically considered to be associated with apoptosis, while the ERK pathway is connected to cell growth and development.

Figure 1 is from an early experiment and shows the differences in IL-6 and IL-8 secretion from HPAEC after stimulation with the two HIV proteins, tat and gp120, at four different concentrations. Figure 2 through Figure 5 are all ELISA results from the same treatment looking at different cytokines. Looking at IL-6 secretion in Figure 1, there are very steady levels across most of the different stimulations. However, the tat (250 ng/mL) dose results in a significant increase in IL-6 secretion, which drops toward normal levels at tat (500 ng/mL). IL-8 secretion is very similar, showing a dramatic increase at tat (250 ng/mL). Interestingly, the drop in IL-8 secretion at tat (500 ng/mL) is much more dramatic than what was seen with IL-6. Initially the decrease in IL-8 secretion with tat (500 ng/mL) was thought to be the result of tat cytotoxicity, but that belief is refuted by other ELISA results that show strong secretion of other cytokines at the same tat concentration (i.e., Figure 4). If the cells were killed, no secretion of these

cytokines would occur.

Figure 2 shows IL-8 concentrations after stimulation by combinations of TNFα, IL-1, tat, and gp120. The gp120 alone has no significant effects, though the tat alone shows some alterations in cytokine profile (see Figure 1). The combinations of tat and gp120 with TNFα and IL-1 show no significant differences when compared with the TNFα and IL-1 control values. This implies that there is no synergistic effect of these inflammatory proteins and HIV proteins in HPAEC on IL-8 secretion. IL-8, an important mediator in leukocyte recruitment and diapedesis as well as in angiogenesis, has been shown to be present in atherectomy samples (Ishibashi et al., 1999; Kato, Matsubara, Iida, Suzuki, Sato, 1999; Simonini et al., 2000). This model shows that if stimulation of these cells is a necessary precursor to plaque formation, the mechanism may not involve IL-8 secretion at this early stage. The presence of IL-8 in plaque may be from a different source, such as invading macrophages and foam cells, or IL-8 may be secreted from endothelial cells only after chronic inflammation.

Mip1a was also assayed as shown in Figure 3. The cells showed no significant differences in Mip1a production when exposed to only the different concentrations of tat and gp120. However, the production of this cytokine when exposed to IL-1 alone was significant and the combination of IL-1 and the HIV proteins showed strong secretion. The IL-1 + gp120 showed very consistent stimulation similar to the IL-1 control. The IL-1 + gp120 showed results similar to the control, but the tat (250 ng/mL) showed a slight decrease and the tat (500 ng/mL) showed a dramatic drop in Mip1a secretion almost to background levels. Again, it does not seem the cells were killed because secretion of

MCP-1 remained consistent in the same experiment (see Figure 4). Apparently the combination of IL-1 and a high dose of tat depresses the secretion of MCP-1 in the HPAEC. The addition of TNFα alone and in combination with tat and gp120 resulted in no significant increases in Mip1a secretion, though there did seem to be some decrease with high doses of TNFα and tat. This pattern shows HPAEC secretion of Mip1a is sensitive to IL-1 but not to TNFα, and that the addition of a high concentration of tat could depress this secretion. Considering Mip1a has been found to be an important HIV suppressing factor from CD8+ T cells, the lack of stimulation by the HIV proteins could be an important factor in plaque, and possibly disease, progression (Cocchi et al., 1995).

The levels of secretion of MCP-1 are shown in Figure 4. There was no increase in MCP-1 secretion by any addition of HIV proteins, but the addition of IL-1 and TNFα were both able to induce strong secretion of MCP-1. When TNFα and IL-1 were added to the HIV proteins, the resulting secretion of MCP-1 was consistently similar to the IL-1 and TNFα controls. The secretion of MCP-1 by HPAEC seems to be almost "all or nothing". When the cells are exposed to the acute phase proteins IL-1 and TNFα, they respond with very strong secretion of MCP-1, but when exposed to the viral proteins, the secretion is very similar to the control.

Secreted IL-6 levels were also measured in the supernatants (Figure 5). An increase in the IL-6 production was seen from stimulation with tat (250 ng/mL), but an even larger increase was seen in tat (500 ng/mL). This was unexpected considering the early results shown in Figure 1 looking at IL-6 secretion at tat (500 ng/mL) showed a different trend. The only other significant difference in production was after treatment to

TNFα (100 U/mL) + tat (500 ng/mL). This increase shows that there is most likely a synergy between TNFα and high levels of tat in IL-6 secretion.

The ELISA results above show that treatment of the tat protein to HPAEC can induce differential cytokine secretion. It is surprising that such little response was seen with the gp120 treatments considering there have been reports of up regulated endothelial cell adhesion molecules and altered blood brain barrier permeability in response to gp120 (Annunziata et al., 1998; Toneatto, Finco, Putten, Abrignani, Annunziata, 1999). Differential cytokine secretion was seen with tat at 250 ng/mL, where there is a dramatic increase in IL-8 production. IL-8 secretion is not enhanced or depressed with costimulation using HIV proteins and TNFα or IL-1. Mip1a can be stimulated using IL-1, but not TNFα, and a surprising drop occurs in Mip1a secretion when IL-1 and high levels of tat are used. MCP-1, like IL-8, shows no difference in regulation when exposed to IL-1 or TNFα and the HIV proteins, nor does any change occur when exposed to the HIV proteins alone. IL-6 levels were increased after treatment to tat (250 ng/mL), but showed even more increase after treatment with tat (500 ng/mL). Also, IL-6 levels increased higher than controls when TNFα and tat (500 ng/mL) were both used. Considering IL-8 and IL-6 have both been present in atherosclerotic plaques it is worth nothing that there seems to be a threshold of tat concentration that must be exceeded before it stimulates production of these cytokines (Kato et al., 1999). Considering these high levels of tat protein are only to be found a very short distance away from areas actively infected with HIV, it is possible that the IL-6 and IL-8 secretion which occurs at the sites of plaque formation are the results of nearby HIV infection. This is also

dependent on numerous factors which can result in plaque formation, including other subclinical infections such as chlamydia and some viruses, family history, lifestyle, vascular flow, and medications. These other conditions could alter the formation of plaques by changing the response of endothelium in different conditions.

From the information above, two possibilities exist for the rapidly advancing state of these plaques in HIV patients. One possibility is that many HIV patients already have a propensity toward plaque formation. Indeed, most Americans have begun to develop fatty streaks, the precursor to plaques, by age 10. HIV patients may have these early plaques subjected to high levels of tat protein by virtue of HIV infection in close proximity to these predisposed areas. This, in turn, could cause an increase in the secretion of atherogenic cytokines, such as IL-6 and IL-8, from that patch of endothelium and dramatically increase the rate of plaque formation. The other possibility is again dependent on the proclivity of the average person today toward heart disease. If this plaque is already forming, then the tendency for invasion of macrophages into the plaque is already present. Because macrophages are subject to infection by the M-tropic strains of HIV through the CCR5 receptor, there is a substantial probability that a few HIV infected macrophages could enter the plaque and become resident. If this were to happen, the concentration of tat protein in the plaque would be increased significantly. IL-6 and IL-8 secretion would increase as a result, and plaque formation may soon be accelerated. Though both theories can stand alone in accounting for the high rate of atherogenesis, there is no reason that both mechanisms could not be involved simultaneously. Ultimately, these alterations could result through changes in oxidation

states, changes in signaling response times, or even alteration of second messenger pathways. Because early results showed the greatest HPAEC response occurred at tat 250 ng/mL (see Figure 1), this concentration was used for further studies to elucidate the second messenger pathways that may be involved in endothelial cell stimulation.

The first pathway analyzed was the NFκB pathway (Figure 6). The results showed that tat can stimulate NFκB to be phosphorylated and cross the nuclear membrane. Considering NFκB is involved in upregulation of genes which result in nitric oxide, cell adhesion molecules, and IκB production, the results mean that tat can be directly responsible for local inflammatory conditions and leukocyte recruitment. The production of inflammatory cytokines and attachment and diapedesis of leukocytes is a necessary early step in plaque formation, and shows that this concentration of tat can be a participant in atherogenesis.

Figure 9 shows MAP kinase p38 phosphorylation at different time points of treatment to known activators of this pathway. This was a necessary first step in determining the amount of time necessary to activate phosphorylation of these proteins. Because the phosphorylation peaked at 45 minutes, this point was chosen for further studies with tat 250 ng/mL. Two important areas of the MAP Kinase cascade were then examined, the p38 and ERK1,2 proteins. The first western blot looked at p38 phosphorylation. The proteins known to be strong activators of this pathway, IL-1 and TNF, both showed significant elevation in band density over the control. But this experiment showed that tat (250 ng/mL) can also stimulate phosphorylation of p38, though not to the extent that IL-1 and TNF can. The other 45-minute treatment with tat

looked at the phosphorylation of ERK1,2, a MAP kinase cascade protein important in cell growth and differentiation (Figures 8,11). The tat treatment (second lane) shows a slight increase in phosphorylation when compared to the control (first lane).

These MAP Kinase results are surprising by what they do not show. Generally, these two pathways are thought of as being opposites: p38 is activated during cellular stress and ERK1,2 is active during cell growth and differentiation. Typically, it is a balance between these two pathways that results in cells performing one way or the other. But this experiment does not show any results that buttress this theory. Instead, it shows that tat can be directly responsible for activating both pathways simultaneously. In so doing, it could explain the observations of Zietz et al. (1996) who saw the increased turnover rate of endothelial cells. If the ERK1,2 pathway is being stimulated by tat, the cells could be undergoing a growth factor-like proliferation response while simultaneously being induced into apoptosis through an activated p38 pathway.

Morphology was compared after tat and gp120 incubations (Figure 12). The control shows normal, attached endothelial cells. After a 24-hour treatment to tat or gp120, however, some changes are seen. The endothelial cells show some detachment from the well, especially at the narrow portions of the cell. Additionally, the cells show increased vacuole formation. The detachment of the cells could be analogous to an increase in permeability between cells seen in other experiments using tat. This would play a role in vascular ability to allow leukocyte diapedesis and, therefore, possibly be important in plaque formation.

The RT-PCR results show qualitatively that RNA for these inflammatory proteins

is being made, and, therefore, the proteins could be made de novo. The missing β-actin band in the fourth lane is probably the result of differential splicing of RNA. By having a slightly different copy of RNA, the primer would not bind and the amplification would not occur. The fact that the bands are very similar in density implies the PCR may have run too many cycles. This experiment used 45 cycles, and it is possible that it amplified each well beyond the point where density differences are still detectable. By running this RT-PCR again with fewer cycles, the results may show a quantitative difference in RNA production between each well.

This research shows that the HIV tat protein, but not gp120, can result in the activation of HPAECs (Figure 14). One way this is achieved is by inducing the phosphorylation and translocation of NFκB. Additionally, the induction of both the p38 and ERK1,2 MAP Kinase pathways could play a role in activation. Once activated by tat, the cells show increases in the secretion of de novo inflammatory proteins, specifically IL-6 and IL-8. As HIV patients are living for longer periods of time with these circulating viral proteins the opportunity for interaction between tat and endothelium increases. The tat stimulated secretion of these atherogenic cytokines as well as the production of cell adhesion molecules through the NFκB pathway could both be of profound importance in the development of atherosclerotic plaques by inducing small areas of chronic inflammation. These regions could be associated with the increased occurrence of atherosclerotic disease in HIV patients. Further research into this area could involve the use of NFκB and MAP Kinase inhibitors to see if their involvement blocks the secretion of inflammatory cytokines. If so, this would show that

these pathways alone are able to respond to tat and result in endothelial activation. Ultimately, by decreasing the long term viral load in the patients the concentration of tat in the circulation should also decrease. This may prove to be the best way to prevent HIV related cardiovascular disease in this patient population.

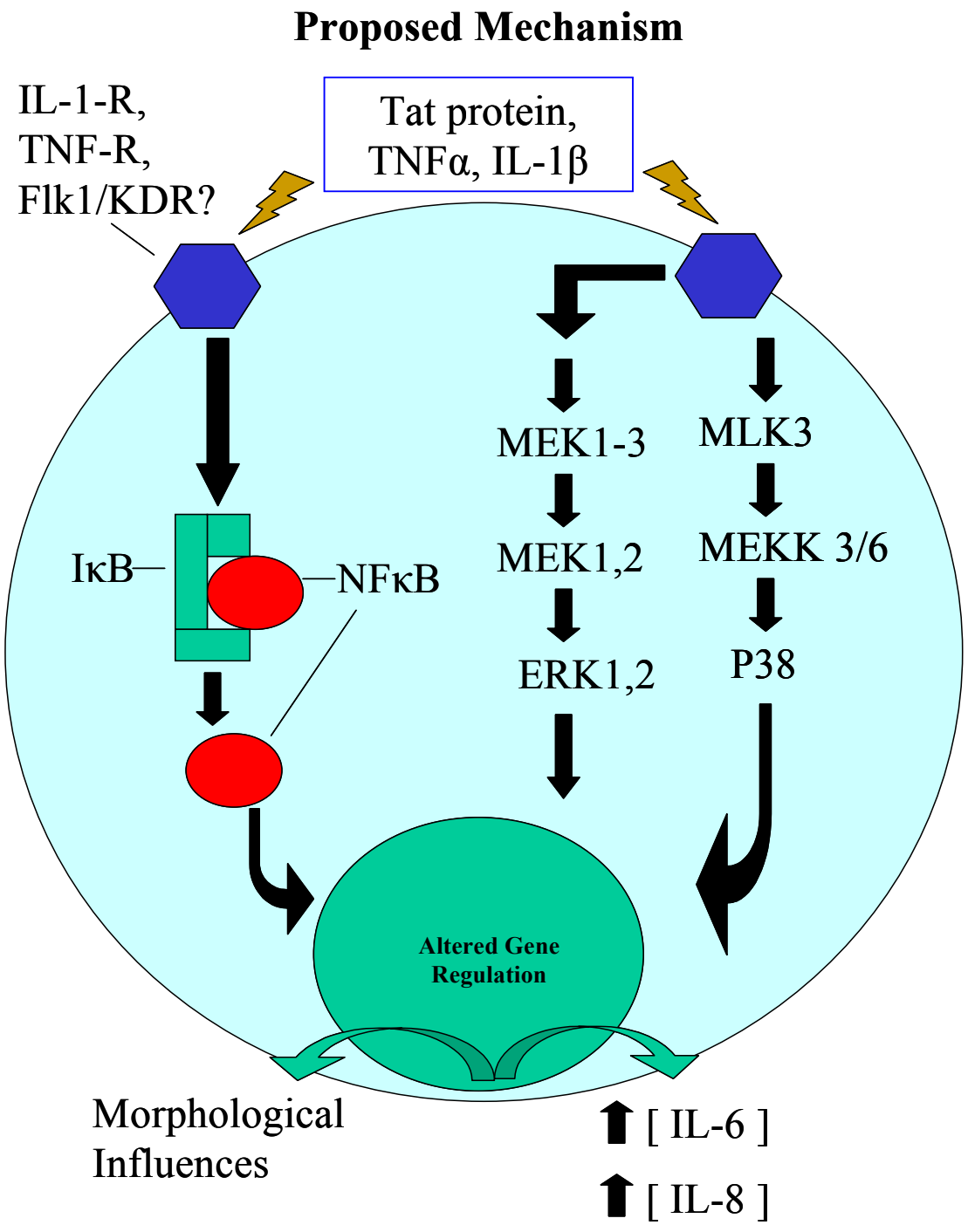


Figure 14: Proposed mechanism based on the findings of this research. See text for discussion.

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VITA

JASON L. HENRY

Personal Data:	Date of Birth: October 13, 1975 Place of Birth: Fort Campbell, KY Marital Status: Single
Education:	Carson Newman College, Jefferson City, TN Biology, BA, 1996
Professional Experience:	Graduate Assistant, East Tennessee State University, College of Medicine, 1999-2000.
Publications:	Kelley, J., Chi, D., Henry, J., et al. HIV and cocaine induced cardiovascular disease: Pathogenesis and clinical implications. *Cardiology Reviews and Reports, July 2000,* 363-370. Chi, D., Henry, J., Kelley, J., et al. The effects of HIV infection on endothelial function. *Endothelium, 7 (4),* 223-242.
Honors and Awards:	U.S. Navy Health Professions Scholarship Recipient, 2000-2004 Medical Class Vice President, 2000-2001 Students' Choice Award, ETSU Student Research Forum, 2000 Inducted Phi Kappa Phi, 2000