Role of 14–3-3ε, c-Myc/Max, and Akt phosphorylation in HIV-1 gp 120-induced mesangial cell proliferation

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Received 17 February 2000; accepted in final form 27 October 2000

Kapasi, Aditi A., Saijun Fan, and Pravin C. Singhal. Role of 14–3-3ε, c-Myc/Max, and Akt phosphorylation in HIV-1 gp 120-induced mesangial cell proliferation. Am J Physiol Renal Physiol 280:F333–F342, 2001.—Focal glomerulosclerosis (FGS) is the predominant glomerular lesion in patients with human immunodeficiency virus (HIV)-associated nephropathy. Initial mesangial cell hyperplasia and subsequent hypoplasia are common features of FGS. In the present study we evaluated the effect of HIV-1 glycoprotein (gp) 120 on human mesangial cell (HMC) growth. HIV-1 gp 120 stimulated HMC proliferation at lower concentrations, whereas it suppressed cell proliferation at higher concentrations. In parallel to the modulation of cell growth, gp 120 at low concentrations resulted in an increase in the expression of c-Myc, Max, and 14–3-3ε proteins and phosphorylation of ATP-dependent tyrosine kinases (Akt) at Ser473. However, the expression of these proteins increased with decreasing concentrations of gp 120. Furthermore, gp 120 also exhibited a dose-dependent inhibition of Akt phosphorylation at Ser473 without any significant alteration of Akt expression. Little or no effects of gp 120 were observed on the expression of extracellular signal-regulated kinase (ERK), phospho-ERK, Bel-2, and Bax proteins. At a higher concentration, gp 120 not only promoted HMC apoptosis but also enhanced expression of Fas and FasL. These results suggest that HIV-1 gp 120 induces alterations in conflicting survival signaling pathways that contribute to the potential dual effects of gp 120 in promoting or inhibiting HMC proliferation.

human immunodeficiency virus-1 glycoprotein 120; human mesangial cells; proliferation; ATP-dependent tyrosine kinases; Myc; Max

HUMAN IMMUNODEFICIENCY VIRUS (HIV)-associated nephropathy (HIVAN) is a renal lesion frequently observed in patients with acquired immune deficiency syndrome. Alterations in mesangial cell growth patterns are important features of glomerular lesions in patients with HIVAN (14, 61). Transgenic mice with subgenomic proviral HIV-1 construct showed progressive renal lesions for glomerulosclerosis that are both clinically and histologically similar to those in patients infected with HIV (43). Moreover, total kidney lysates from these transgenic mice showed a peptide that is recognized by antiserum specific for HIV-1 envelope glycoprotein (gp) 160 (25). We have previously found that HIV-1 gp 160 modulated mesangial cell proliferation and apoptosis (66). We hypothesize that HIV-1 gp 120, a major component of gp 160 protein, may be activating human mesangial cells (HMC) to proliferate under certain conditions; however, gp 120 may also be suppressing the growth of HMC under different conditions. Previously, gp 120 has been reported to suppress the growth of kidney fibroblasts through the induction of apoptosis (64). Apoptosis is a regulated way by which cells delete unwanted cells. If it is physiological, it maintains the homeostasis; however, when it is pathological, it may compromise the function of affected cells. For example, in a diseased state bacteria can compromise the host’s defense by inducing monocyte apoptosis. Similarly, HIV is known to induce immune deficiency through the induction of CD4+ T cell apoptosis. On the other hand, neutrophils normally die through apoptosis without inducing any inflammation.

The interaction of gp 120 with the CD4 receptor by forming a one-to-one complex is the initial event in viral entry into the host (6, 11, 21) and plays a potentially important role in inducing functional immunosuppression after infection with HIV (17). Moreover, CD4 not only serves as a receptor for HIV but also contributes to specific cell-cell adhesion in humans (17, 31, 57, 67). We and other investigators have demonstrated that human renal tubular epithelial cells express CD4 receptors (22, 36–40).

In the present study, we have investigated the effect of gp 120 on proliferation of HMCs and the mechanisms of gp 120-mediated cell proliferation. Our results indicate that gp 120 has both growth stimulating and inhibiting activities in HMCs. At lower doses, gp 120-stimulated cell proliferation was not only associated with an increased expression of 14–3-3 ε, c-Myc, and its partner Max but also increased phosphorylation of ATP-dependent tyrosine kinases (Akt) at Ser473 whereas increased concentrations of gp 120 caused suppression of cell growth in addition to a significant decrease in expression of 14–3-3ε, c-Myc/Max, and Akt phosphorylation. These findings suggest that gp 120-
induced gene expression programs are the molecular basis for conflicting signaling pathways to stimulate or inhibit proliferation of HMCs.

**MATERIALS AND METHODS**

**Cell culture and gp 120.** HMCs were obtained from Clo- netics (San Diego, CA) and maintained in RPMI-1640 supple- mented with 10% heat-inactivated fetal calf serum (FCS), 2% penicillin and streptomycin, 1% HEPES, 2 mg sodium bicarbonate, and 2 mM L-glutamine in an environment of 5% CO₂-95% air at 37°C. All experiments were performed in HMCs between passages 5 and 8. HIV-1IIIB gp 120 peptide (18, 58), anti-HIV-1 gp 120 monoclonal antibody (59), Gag, and Nef were obtained from the National Institutes of Health (NIH; HIV-1 IIIB gp 120 peptide (295–328); anti-HIV-1 gp 120 monoclonal antibody (F-105); Gag, HIV-1 III-B p55 Gag, no. 3276; and Nef, HIV-1LAV Nef, no. 1640; NIH Research and Reagent Program, Rockville, MD).

**Western blot analysis for CD4.** HMCs were cultured in 75-cm² tissue culture flasks, washed with ice-cold PBS, and then fixed with 100% methanol at −20°C. The cells were washed with PBS and incubated with 1.5% normal goat serum for 20 min at room temperature (RT), followed by incubation with rabbit polyclonal anti-human CD4 antibody (T4–4, 1:8,000, NIH Research and Reagent Program) overnight at 4°C. Unbound antibody was completely washed with PBS, and the cells were further incubated with biotinylated goat anti-rabbit antibody (1:200, Vector Labs, Burlingame, CA) for 1 h at RT. The cells were washed with PBS and incubated with the avidin-biotin complex (Vectastain Elite ABC kit, Vector Labs) for 30 min followed by incubation with 0.1% dianibenzoazinedetetrachloride (Sigma, St. Louis, MO) in 0.1 M Tris buffer, pH 7.6, containing 0.02% H₂O₂ for 1–2 min at RT. Finally, the slides were rinsed with tap water followed by distilled water and mounted with coverslips using Permount (Fisher Scientific, Pittsburgh, PA). Negative controls were performed using the same procedure, except that nonspecific rabbit IgG was used instead of CD4 primary antibody.

The above staining was also repeated by using goat polyclonal anti-CD4 (C-18) antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

**Western blot analysis for CD4.** HMCs were cultured in 75-cm² tissue culture flasks, washed with ice-cold PBS, and lysed in ice-cold RIPA lysis buffer containing protease inhibitors (Calbiochem, San Diego, CA) and 100 mM sodium orthovanadate. The cell lysates were transferred through a syringe fitted with a 21-gauge needle to a microcentrifuge tube to shear the DNA and incubated for 1 h on ice. Phenylmethylsulfonyl fluoride (10 mg/ml) in isopropanol was added to the cell lysate and incubated for another hour on ice. The cell lysates were centrifuged at 15,000 g for 20 min at 4°C. The supernatant was collected and measured for protein content using the Bio-Rad dye-binding microassay (Bio-Rad Laboratories, Hercules, CA). A portion of total protein lysates (100 µg/lane) was electrophoresed on 5% polyacrylamide (PAGE) and transferred onto a polyvinylidene difluoride membrane by electroblotting. A colored marker (Bio-Rad Laboratories) was used as a molecular-size standard. The membranes were further incubated with goat polyclonal antibody for anti-CD4 (C-18) (200 µg/ml, Santa Cruz Biotechnology) and then with horseradish peroxidase-labeled secondary donkey anti-goat antibody (Santa Cruz Biotechnology). Blots were developed by using enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL).

**Proliferation studies.** Proliferation of HMCs was determined by using either trypan blue dye exclusion assay or 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. For the trypan blue dye exclusion assay, cells (1 × 10⁴ cells/well) plated in 24-well tissue culture plates were grown to subconfluence. Cells were washed twice with PBS and growth-arrested in medium with additional 5% insulin-transferrin-selenium (ITS), and 0.5% BSA (GIBCO, Gaithersburg, MD) for 36 h. The cells were again washed twice with PBS and incubated in 500 µl of medium (RPMI-1640+1% FCS) containing either vehicle (control), HIV-1 gp 120 (0.00001–0.1 µg/ml), anti-gp 120 antibody (2.5 µg/ml), or HIV-1 gp 120 (0.00001 µg/ml)+anti-gp 120 antibody (2.5 µg/ml) overnight at 37°C. At the end of the scheduled incubation period, cells were harvested with trypsin and stained with 0.4% trypan blue. The trypan blue-positive and -negative cells were counted by using a hemocytometer under phase-contrast microscopy. For the MTT assay, 2 h after the trypan blue dye exclusion assay, cells (2 × 10⁴ cells/well) plated in 96-well tissue culture dishes were growth-arrested and treated with various doses of gp 120 as described above. MTT (2 mg/ml; Sigma Chemical) was added into each well for 4 h. After 4 h, the medium containing MTT was aspirated from the wells, and the formazan crystals were dissolved in 100 µl of 0.04 N HCl in isopropanol. The absorbance was recorded in an ELISA reader at 550 nm, with 620 nm as the reference wavelength. Wells that contained only medium and 10 µl of MTT were used as blanks for the plate reader. Four sets of experiments were performed in triplicate for each treatment. The results were analyzed by a Student-Newman-Keuls multiple comparison test (2-tailed) with normal approximation.

**Effect of Gag and Nef on HMC cell proliferation.** The effect of other HIV-1 peptides such as Gag and Nef on proliferation of HMCs was determined by using either a trypan blue dye exclusion assay or MTT assay. For both assays, cells plated in 24-well (trypan blue dye exclusion assay) and 96-well (for MTT assay) tissue culture plates were cultured to subconfluence. Cells were washed twice with PBS and growth-arrested in medium with additional 5% ITS, and 0.5% BSA (GIBCO) for 36 h. The cells were again washed twice with PBS and incubated in 500 µl of medium (RPMI-1640+1% FCS) containing either vehicle (control) or HIV-1 Gag and Nef (0 and 0.00001 µg/ml) for overnight at 37°C and harvested for determination of cell number using a trypan blue dye exclusion assay and MTT assay as mentioned earlier.

**Western blot assay.** Growth HMCs in RPMI 1640 with 5% ITS and 0.5% BSA (36 h) were washed twice with PBS and treated with HIV-1 gp 120 (0–0.1 µg/ml) for 16 h. Cells were then washed with ice-cold PBS and lysed in a minmodified ice-cold RIPA lysis buffer containing protease inhibitors (Calbiochem) and 100 mM sodium orthovanadate. The cell lysates were transferred through a syringe fitted with a 21-gauge needle to a microcentrifuge tube to shear the DNA and incubated for 1 h on ice. Phenylmethylsulfonyl fluoride (10 mg/ml) in isopropanol was added to the cell lysate and incubated for another hour on ice. The cell lysates were centrifuged at 15,000 g for 20 min at 4°C. The supernatant was collected and measured for protein content using the Bio-Rad dye-binding microassay (Bio-Rad Laboratories, Hercules, CA). A portion of total protein lysates (100 µg/lane) was electrophoresed on 5% polyacrylamide (PAGE) and transferred onto a polyvinylidene difluoride membrane by electroblotting. A colored marker (Bio-Rad Laboratories) was used as a molecular-size standard. The membranes were further incubated with goat polyclonal antibody for anti-CD4 (C-18) (200 µg/ml, Santa Cruz Biotechnology) and then with horseradish peroxidase-labeled secondary donkey anti-goat antibody (Santa Cruz Biotechnology). Blots were developed by using enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL).
RESULTS

**Immunocytochemical staining for CD4 receptor expression.** We used immunocytochemical staining to identify the expression of CD4 receptors in HMCs. As shown in Fig 1A, HMCs showed immunocytochemical staining for CD4 receptors using a rabbit polyclonal anti-human CD4 antibody (T4-4). The cells were stained with normal rabbit serum as a negative control and are shown in Fig 1B. These results indicate that HMCs carry CD4 receptors. The present studies showing the expression of CD4 are in agreement with our previous studies and with those of others (22, 36–40).

**Effect of HIV-1 gp 120 on cell proliferation.** HIV-1 envelope gp 120, a major component of HIV-1 gp 160, is known to participate in viral entry into the host via its specific receptor binding. HMCs used in our present studies contain CD4 receptors as determined by immunocytochemical staining and Western blotting for CD4 receptors. To study the effect of HIV-1 gp 120 on cell proliferation, HMCs were plated in 24-well tissue culture plates and then treated with gp 120 in different doses (0–0.1 μg/ml) at a fixed time interval. Finally, cell number and viability were analyzed by using either a trypan blue dye exclusion assay or MTT assay. Similar results were observed for both assays, as illustrated in Fig. 2. There were two distinct responses to HIV-1 gp 120 in HMC growth: at lower concentrations, gp 120 promoted and, at higher concentrations, suppressed the proliferation of HMCs. Gp 120 at a concentration of 0.00001 μg/ml stimulated (P < 0.001) cell proliferation; whereas gp 120 at a concentration of 0.01 μg/ml attenuated (P < 0.001) cell growth compared with untreated cells (control). To exclude the possibility that HMCs may have accumulated specific mutations during passage, or other potential passage effects, similar studies were also performed in HMCs with different passages (data not shown), which gave results identical to the data shown in Fig. 2.

**Effect of anti-gp 120 antibody on gp 120-induced cell proliferation.** To study whether the effect of HIV-1 gp 120 on cell proliferation is blocked by anti-gp 120 antibody, HMCs were plated in 24-well tissue culture plates and then treated with gp 120 (0 and 0.00001 μg/ml) with or without anti-gp 120 antibody (2.5 μg/ml) at a fixed time interval. Subsequently, cell number and viability were analyzed by using either a trypan blue dye exclusion assay or MTT assay. Anti-gp 120 inhibited the effect of gp 120 by 50% (Table 1).

**Effect of Gag and Nef on HMC cell proliferation.** To study whether there is any effect of other HIV-1 peptides on HMC proliferation, HMCs were plated in 24-well tissue culture plates and then treated with Gag and Nef (0 and 0.00001 μg/ml) at a fixed time interval. Subsequently, cell number and viability were analyzed by using either trypan blue dye exclusion assay or MTT assay. Neither Gag nor Nef modulated the proliferation of HMCs (Table 2).

**Effect of HIV-1 gp 120 on HMC apoptosis.** To determine the effect of gp 120 on HMC apoptosis, equal numbers of HMCs were incubated in media containing either buffer (control) or gp 120 (0.001, 0.01, 0.1 μg/ml) for 16 h. Four sets of experiments were carried out. As shown in Table 3, gp 120 promoted (P < 0.001) HMC apoptosis in a dose-dependent manner.

**Effect of gp 120 on cell proliferation-associated proteins.** To understand the molecular mechanism of the modulation of cell proliferation by gp 120, we examined the effect of gp 120 on the expression of several proteins related to cell proliferation. In parallel to the study of cell proliferation described above, HMCs were...
Fig. 1. Expression of CD4 in HMCs. A: expression of CD4 receptor in human mesangial cells (HMCs) by immunocytochemistry. HMCs plated on chamber slides were cultured to subconfluence, permeabilized with methanol, and processed for immunocytochemical staining. Sets of representative photomicrographs are shown. a: HMCs stained with a specific antibody for CD4 receptor expression. b: HMCs stained with normal rabbit serum (negative control). B: expression of CD4 receptor in HMCs by Western blotting. Confluent HMCs were scraped and dissolved in RIPA buffer containing protease inhibitors, separated on PAGE, probed with a specific antibody to CD4, blotted with horseradish peroxidase-labeled anti-goat IgG, and detected by enhanced chemiluminescence (ECL).

Fig. 2. Effect of glycoprotein (gp) 120 on proliferation of HMCs. HMCs were treated with gp 120 at indicated doses for 16 h and harvested for determination of cell number by using a trypan blue dye exclusion assay (A) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (B). HIV-1, human immunodeficiency virus-1. *P < 0.001 compared with untreated control and gp 120, 0.0001 μg/ml. **P < 0.001 compared with untreated control and gp 120, 0.001 μg/ml. ***P < 0.05 compared with the untreated control.
growth-arrested and treated with different doses of gp 120. Total cell proteins were then extracted and subjected to Western blotting.

A set of Western blots is shown in Fig. 3A. The bands of these proteins were quantitated by densitometry and expressed as relative to the actin bands (Fig. 3B). Expression of 14–3-3 protein was increased after treatment with 0.00001 μg/ml gp 120, reached a maximum (∼2-fold) at 0.0001 μg/ml, and then started to decline with increasing concentrations of gp 120 and finally dropped to ∼20% of untreated control level at 0.1 μg/ml. Expression of c-Myc protein was increased after exposure to 0.00001 μg/ml and markedly decreased with increasing concentrations of gp 120 to undetectable levels at 0.01 μg/ml. Similar results were also obtained for the expression of Max protein. Max protein contains two isoforms, MaxL (22 kDa) and Maxs (21 kDa), as determined by using Max polyclonal antibody (C-124). MaxL and Maxs followed the same pattern in response to gp 120.

Using specific antibodies for Akt and the phosphorylated form of Akt at Ser^473 (phospho-Akt), we determined the effect of gp 120 on Akt expression. As illustrated in Fig. 3, HMCs expressed both total Akt protein and the phospho-Akt form. However, at lower doses it induced phosphorylation of Akt at Ser^473. The phosphorylation of Akt was hard to detect at 0.001 μg/ml. Similarly, gp 120 at low doses (0.00001 and 0.0001 μg/ml) slightly increased the phosphorylation of ERK protein and, at high concentrations (0.001 and 0.01 μg/ml), it decreased the phosphorylation of ERK protein (<20% decrease). Two close phosphorylation forms were determined by phospho-ERK antibody (E10 monoclonal antibody, New England Biolabs), suggesting that HMCs have phospho-p44/42 mitogen-activated protein (MAP) kinases (Thr^202/Tyr^204) in our experimental conditions. Again, gp 120 did not exhibit a significant effect on the expression of total ERK protein, except at the highest dose (0.1 μg/ml), which caused a 27% decrease. There was no alteration in actin protein, which acts as a control for the determination of equal protein loading.

Effect of gp 120 on cell death-associated proteins. Proapoptotic factors such as Fas and FasL have been demonstrated to play an important role in the induction of apoptosis. Because glomerular cells in culture have been reported to express Fas and FasL (54), we examined whether gp 120 at higher concentrations could also modulate HMC expression of Fas and FasL. At higher concentrations, gp 120 enhanced the expression of both Fas and FasL (Fig. 4). In addition, we determined whether gp 120 at higher doses modulated the expression of Bcl-2 and Bax, proteins often required for the modulation of apoptosis (7). There was no significant change in the expression of Bcl-2 or Bax proteins after exposure to gp 120 at higher doses (Fig. 3). Our previous studies suggested that gp120 at higher concentrations suppressed renal fibroblast growth (64); we believe that similar mechanism may also be playing a role in gp 120-induced fibroblast apoptosis.

DISCUSSION

HIV-1 envelope glycoprotein gp 120 binds to the CD4 receptor and mediates the fusion of biological membranes; this results in subsequent events leading to syncytium formation, signaling cascade, cytopathic effects, and priming of cells for cell suicide, or death (17, 20, 21, 31, 57, 67). Various investigators have shown correlation of viral load and CD4 count (34, 68). In addition, these investigators also demonstrated that severity of HIV infection also correlates to low CD4 count. Because gp 120 is

Table 1. Effect of anti-gp 120 antibody on proliferation of HMCs by trypan blue dye exclusion and the MTT method

<table>
<thead>
<tr>
<th>Method</th>
<th>Control</th>
<th>Anti-gp 120 Antibody</th>
<th>Gp 120</th>
<th>Gp 120 + Anti-gp 120 Antibody</th>
</tr>
</thead>
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<tr>
<td></td>
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<td></td>
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<tr>
<td>Trypan blue</td>
<td>1.2 × 10^5 ± 3.9 × 10^5</td>
<td>1.1 × 10^5 ± 23.3 × 10^5</td>
<td>2.9 × 10^5 ± 9.2 × 10^5</td>
<td>1.5 × 10^5 ± 14.8 × 10^5</td>
</tr>
<tr>
<td>MTT</td>
<td>1.3 × 10^5 ± 3.9 × 10^5</td>
<td>1.1 × 10^5 ± 20.1 × 10^5</td>
<td>2.8 × 10^5 ± 9.2 × 10^5</td>
<td>1.5 × 10^5 ± 14.8 × 10^5</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as cell no./ml. HMCs were treated with vehicle (control), glycoprotein (gp) 120 (0.00001 μg/ml), anti-gp 120 antibody (2.5 μg/ml), and a combination of (gp 120 + anti-gp 120 antibody) for 16 h and harvested for determination of cell number by using a trypan blue dye exclusion and MTT assay. *P < 0.001 compared with untreated control (with and without) anti-gp 120 antibody and gp 120 (0.00001 μg/ml) with anti-gp120 antibody.

Table 2. Effect of HIV-1 Gag and Nef on proliferation of HMCs by a trypan blue dye exclusion and the MTT method

<table>
<thead>
<tr>
<th>Trypan Blue Dye Exclusion</th>
<th>MTT</th>
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<tbody>
<tr>
<td>Gag</td>
<td>Nef</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.2 × 10^5 ± 1.1 × 10^5</td>
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<tr>
<td>0.00001, μg/ml</td>
<td>1.4 × 10^5 ± 0.4 × 10^5</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as cell no./ml. HMCs were treated with vehicle (control), Gag and Nef (0.00001 μg/ml) for 16 h and harvested for determination of cell number by using a trypan blue dye exclusion and MTT assay. HIV, human immunodeficiency virus.
a component of the HIV-1 virus, it is likely to be high during the increased viral load or in the later stages of the disease. Therefore, it is possible that increased viral load in the later period of the disease may also be contributing to the mesangial cell hypoplasia. However, it is not plausible to extrapolate correlation of in vivo findings to our in vitro data. At best, we can infer that our in vitro findings are consistent with the natural course of HIVAN.

Previous reports have shown the expression of CD4 receptors at detectable levels by immunocytochemical staining (38, 39) and by flow cytometry (22) in HMCs. HIV-1-infected mesangial cells in vitro and glomeruli isolated from HIV-1-infected patients contain HIV-1 genomic DNA (29, 42). In previous studies, we also found that HIV-1 gp 160, sera from HIV-infected patients, and culture supernatants of HIV-1 infected cells altered the phenotypes of mesangial cells (51, 63, 66). Furthermore, HIV-1 envelope protein, gp 160, trig-

Table 3. Dose-response effect of gp 120 on HMC apoptosis

<table>
<thead>
<tr>
<th>HIV-1 gp 120, μg/ml</th>
<th>0.001</th>
<th>0.01</th>
<th>0.1</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.21 ± 0.09%</td>
<td>6.3 ± 0.3%*</td>
<td>10.8 ± 0.8%+†</td>
</tr>
<tr>
<td></td>
<td>20.6 ± 0.8%+‡</td>
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</table>

Values are means ± SE expressed as % apoptotic cells/well. HIV-1, human immunodeficiency virus-1. *P < 0.001 compared with untreated control. †P < 0.001 compared with 0.001 μg/ml. ‡P < 0.001 compared with 0.001 and 0.01 μg/ml.

Fig. 3. Effect of gp 120 on proteins associated with HMC proliferation. A: growth-arrested HMCs were washed twice with PBS and treated with HIV-1 gp 120 (0–0.1 μg/ml) for 16 h and then harvested for Western blotting as detailed in MATERIALS AND METHODS. Equal aliquots of total protein (100 μg/lane) were analyzed on 10–15% SDS-polyacrylamide gels and blotted to detect different proteins. Actin was detected on the same gel as a control of protein loading and transfer. ERK, extracellular signal-regulated kinase; phosph, phospho; Akt, ATP-dependent tyrosine kinases. B: protein bands were quantitated by densitometry and expressed relative to the actin bands.
gered apoptosis of mesangial cells at higher concentrations (22) and proliferation at lower concentrations (66).

In agreement with these previous observations, our present study also demonstrates that renal HMCs possess CD4 receptors in culture. HIV-1 gp 120 protein, through binding to CD4, enhanced the proliferation of HMCs at lower concentrations (0.00001 and 0.001 μg/ml). Moreover, gp 120 at these doses significantly increased the expression of cell growth-related proteins, 14–3-3e, c-Myc, and Max. In addition, gp 120 at 0.00001 μg/ml enhanced the phosphorylation of Akt at Ser473. In contrast to the phenotypes at lower doses, gp 120 at higher doses markedly inhibited the cell proliferation, accompanied by a downregulation of these proteins and a decrease in phosphorylation of Akt at Ser473. At higher concentrations (0.001, 0.01, and 0.1 μg/ml), gp 120 not only promoted HMC apoptosis but also enhanced the expression of Fas and FasL. Despite the distinct effects on cell proliferation, gp 120 had little or no effect on the expression of bcl-2, Bax, total Akt, and ERK proteins. These findings indicate that the inhibitory effect of HIV gp120 on HMC growth is associated with a significant reduction in 14–3-3e and c-Myc/Max expressions. This was not due to a general inhibition of protein synthesis by gp 120 because we could not detect any change in the expression of bax and bcl-2. Because the effects of gp 120 on 14–3-3e, c-Myc/Max, and Akt phosphorylation are very similar or identical, it appears that these effects may be mediated through parallel pathways. Thus our present study is the first observation demonstrating involvement of 14–3-3e, c-Myc/Max, and Akt signaling pathways, which are involved in HIV gp 120-modulated cell proliferation of HMCs.

**Fig. 5.** Model for gp 120 activity on proliferation of HMCs. See text for a description.
Besides c-Myc/Max, 14–3-3 protein is a family of signaling intermediates composed of at least seven mammalian isoforms and consists of highly conserved acidic proteins that are expressed in a broad range of tissues and cell types (1, 53). There is increasing evidence that 14–3–3 proteins are important regulators in cell transformation and mitogenic signaling (46). Moreover, it was found that the 14–3–3ε protein interacts directly with calmodulin and participates in signal transduction and cell proliferation (47).

The c-Myc oncoprotein is an important member of the basic helix-loop-helix-leucine zipper family of transcription factors and is involved in cellular processes such as proliferation, transformation, differentiation, and apoptosis (5, 26). Deregulated c-Myc expression is a potent mechanism in apoptosis induction after deprivation of growth factors or forcible growth-arrest with a cytostatic drug (55). Thus the induction of c-Myc in cells grown in the presence of appropriate growth factors or coexpression of other survival genes, such as bcl-2, promoted cell proliferation. However, in the absence of these factors, c-Myc expression causes cell death (55). Many biological properties of c-Myc have been attributed to its association with Max (3, 4, 8). Max (two isoforms, MaxL and MaxS) is another basic helix-loop-helix-leucine zipper protein that heterodimerizes with c-Myc and plays a central role in the transcriptional control of Myc oncoproteins (9, 60, 69). These heterodimers bind DNA strongly and activate transcription (4). However, because Max lacks a transactivation domain, Max overexpression has been reported to repress the transcription of genes bearing c-Myc binding sites (15, 30, 44). Because Max plays a central role in both the positive and negative regulation of c-Myc activity, such opposing effects may provide a more sensitive control over cell growth regulation (4–6). The provocative observation we made in this report is that gp 120 can mediate HMC proliferation through coordinate up- and downregulation of c-Myc and Max protein expressions.

The Fas molecule has been implicated in the induction of high levels of lymphocyte apoptosis seen in HIV-infected patients (19). Interestingly, in our studies, too, gp 120 not only caused apoptosis of HMCs but also enhanced the expression of both Fas and FasL. These findings suggest that Fas as well as FasL may play a role in HMC apoptosis.

Akt, also referred to as PKB and RAC, is the cellular homolog of the viral oncogene v-Akt (16, 27, 28). This protein kinase can be activated by insulin and various growth factors and functions in a pathway involving phosphatidylinositol 3-kinase (PI3 kinase) (16, 27). Recent evidence suggests that Akt functions to promote cell survival by actively inhibiting apoptosis, although the pathways involved have not yet been identified (28, 41). The activation of signal transduction pathways by growth factors, hormones, and neurotransmitters is mediated through two closely related MAP kinases, p44 and p42, designated ERK1 and ERK2, respectively (12, 13, 32). Both p44 and p42 MAP kinases function in a protein kinase cascade that plays a critical role in the regulation of cell growth and differentiation (23, 33, 35, 51).

In summary, we have proposed and examined a hypothesis in the present study in which gp 120 simultaneously activated both positive and negative signaling pathways responsible for the promotion and inhibition of HMC proliferation, as illustrated in Fig. 5. However, the upstream gp 120 signaling pathways involved in these processes remain to be established. Further studies in our laboratory on HMCs transfected with vectors expressing active Akt and c-Myc are in progress to shed more light on pathways responsible for promoting and inhibiting HMC proliferation.

This work was supported by National Institute on Drug Abuse Grant R01 DA-12111.

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