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Original Articles

Interleukin-6 and Glucocorticoids Synergistically Induce Human Immunodeficiency Virus Type-1 Expression in Chronically Infected U1 Cells by a Long Terminal Repeat Independent Post-Transcriptional Mechanism

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Abstract

Background: Glucocorticoids (GC) such as dexamethasone (Dex) can directly upregulate human immunodeficiency virus type-1 (HIV-1) replication in acutely infected cells and potentiate HIV expression from chronically infected promonocytic U1 cells stimulated with tumor necrosis factor-α (TNF-α). We have here investigated the potential effect of Dex in U1 cells stimulated with interleukin-6 (IL-6), a cytokine inducing virus expression by acting mostly at a post-transcriptional level on the virus life cycle.

Materials and Methods: Virus production in culture supernatants was evaluated by reverse transcriptase (RT) activity. GC receptor expression was tested by both binding of [^{3}H]-Dexamethasone 21-mesylate and Northern blotting. Cell-associated HIV protein expression was analyzed by Western blotting, whereas both HIV and monocyte chemoattractant protein-1 (MCP-1) RNA accumulation were evaluated by Northern blotting. HIV transcription was tested by long terminal repeat (LTR) chloramphenicol acetyl transferase (CAT) assay after transient transfection of U1 or U937 cells. Formation of activating protein-1 (AP-1) DNA binding complex in nuclear cell extracts was visualized by electrophoretic mobility shift assay (EMSA), whereas ERK1/2 mitogen-activated protein kinase (MAPK) phosporylation was studied by Western blotting.

Results: IL-6 and Dex synergistically induced HIV expression in U1 cells, and this effect was blocked by RU 486. No substantial HIV RNA accumulation was demonstrated in U1 cells co-stimulated with IL-6 and Dex, whereas IL-6 upregulated the expression of MCP-1 RNA, and this effect was inhibited by Dex. In contrast, Dex potentiated IL-6 induced activation of AP-1 and ERK1/2 MAPK phosphorylation, as revealed by EMSA. HIV-1 LTR driven transcription was observed in U1 cells stimulated with TNF-α and this effect was potentiated by Dex. In sharp contrast, no induction of LTR-directed CAT activity was observed in transfected U1 cells (or in their parental uninfected U937 cells) stimulated with IL-6 and Dex either alone or in combination.

Conclusions: High levels of virion production can be induced in latently infected cells by stimulation with IL-6 and Dex in the absence of activation of the HIV LTR or viral transcription in spite of activation of both ERK1/2 MAPK and AP-1. These findings suggest the existence of LTR-independent pathways influenced by cytokine and GC through which HIV can maintain substantial levels of protein expression and virion production.

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Introduction

The capacity of the human immunodeficiency virus type-1 (HIV-1) to replicate in immune cells, mostly CD4$^+$ T lymphocytes and mononuclear phagocytes, is a major determinant of disease progression (1). Various host factors have been found to modulate viral

replication *in vitro*, including several cytokines and glucocorticoid hormones (GC) that have been previously described as important determinants. In particular, pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interferon-γ, and IL-6 have been shown to upregulate HIV replication in a variety of *in vitro* model systems. Conversely, anti-inflammatory cytokines including IL-4, IL-10 and transforming growth factor-β can either suppress or activate virus expression as a function of the experimental conditions, as reviewed (1,2).

GC are important mediators of the hypothalamus-pituitary-adrenal (HPA) axis that is activated as a feedback system in response to inflammation (3). The involvement of the HPA axis in HIV disease has been associated with either increased or decreased levels of cortisol, particularly after combination therapy including protease inhibitors (4–8). *In vitro*, GC have been reported to induce HIV replication. Early studies indicated that addition of high concentrations of hydrocortisone to co-cultures of peripheral blood mononuclear cells (PBMC) of infected individuals with mitogen-stimulated allogeneic PBMC increased the frequency of virus isolation and/or induced higher levels of virus production compared to GC-untreated co-cultures (9,10). A putative GC responsive element was early identified in the HIV-1 long terminal repeats (LTR) (11–14) and evidence of upregulation of virus production have been obtained in both chronically infected cell lines (15,16) and monocyte-derived macrophages (17,18).

Although GC are classic anti-inflammatory agents that suppress the synthesis and release of pro-inflammatory cytokines and chemokines (19–25) synergistic effects between GC and certain cytokines, including IL-6, have been observed (26–31). In this regard, we have reported that treatment of chronically infected U1 cells with physiologic concentrations (10^{-8} M) of GC alone did not induce virus production, but strongly potentiated the induction of HIV expression by TNF-α (32), a cytokine which stimulates HIV transcription driven by the activation of the cellular transcription factor NF-kB that binds to its long terminal repeats (LTR) (33). The present study investigated the effect of GC on HIV expression induced by IL-6, a cytokine that upregulates HIV expression from U1 cells by acting mostly at a post-transcriptional level (34).

Materials and Methods

U1 Cell Line and Reagents

The U1 cell line was derived from U937 promonocytic cells surviving the cytopathic effect associated with the acute infection by HIV-1LAI/IIIB (35,36). U1 cells contain two integrated copies of proviral HIV DNA and are characterized by low constitutive levels of virus expression that can be upregulated by several cytokines and phorbol esters (37,38). U1 cells (2×10^5 cells/ml) were resuspended in RPMI 1640 (M.A. Whittaker Bioproducts, Walkersville, MD) supplemented with 1 mM HEPES buffer, antibiotics and glutamine plus 10% fetal calf serum (FCS) (Hyclone Laboratories, Logan, UT), containing less than 7 pg/ml of endotoxin. The cells were cultured in 96 or 48 well flat-bottomed plastic plates (Costar, Cambridge, MA) and incubated with the different stimuli at 37°C in 5% CO_2. Mifepristone (RU 486), a pharmacological compound blocking the binding of GC and progesterone to their cytoplasmic receptors (39), was kindly provided by Dr. Stoney Simons, National Institutes of Health, Bethesda. U1 cells were stimulated with TNF-α (100 U/ml; Genzyme Corp., Boston, MA), IL-6 (100 U/ml; R & D Systems), Dexamethasone (Dex) (10^{-8} M; Sigma Chemical Corp., St. Louis, MS), alone or in combination, in the presence or absence of RU 486 (10^{-6} M).

Glucocorticoid Receptor Binding Assay

Cytosol was prepared by washing 2×10^7 U1 cells 3 times in PBS and by freezing the cell pellet on dry ice. All reactions from this point on were performed at 4°C. Receptor binding was assayed as described (40) with minor modifications. The pellets were thawed by adding 400 μl of binding buffer (25 mM HEPES, pH: 7.8, 20 mM Na_2MoO_4, 1 mM EDTA, 10% glycerol). The suspension was vortexed, mixed, and cellular debris was removed by centrifugation in a microfuge (Eppendorf, Hamburg, Germany). An aliquot (95 μl) of cytosol was labeled with [^{3}H]-Dex 21-mesylate (37 Ci/mmole, New England Nuclear, Wilmington, DE) in the presence or absence of an excess of [^{1}H]-Dex. In order to remove the unbound steroid, a 10% solution of dextran-coated charcoal suspension was added. The precipitate was centrifuged and the number of bound receptors was determined by counting aliquots of the supernatants on a scintillation counter.

Reverse Transcriptase (RT) Activity Assay

Thirty μl of culture supernatants were collected at the indicated days after stimulation of U1 cells and were stored at −80°C until tested for the presence of RT activity, as previously described (32,34). Briefly, 5 μl of U1 supernatants were added, in duplicate, to 25 μl of a mixture containing poly (A), oligo (dT) (Pharmacia, Piscataway, NJ), $MgCl_2$ and [^{32}P]-labeled deoxythymidine 5′-triphosphate (dTTP) (Amersham, Arlington Heights, IL) and incubated for 2 h at 37°C. Then, six μl of the mixture were spotted onto DE81 paper (Whatman International, Maidstone, UK), air-dried, washed five times in 2× standard saline citrate buffer, and two additional times in 95% ethanol. The paper was then dried, cut, and counted in a Beckman LS 5000 scintillation counter.

Western Blot of Cell-associated HIV Proteins

Twenty μl from the lysate of 10^7 cells were added to each lane and subjected to electrophoresis through

10–20% gradient polyacrylamide gels (Integration Separation Sci., Hyde Park, MA) for 6 h. The migrated proteins were then transferred overnight onto nitrocellulose filters. After saturation with a 5% milk solution, filters were incubated for 2 h with 1:1,000 (vol/vol) dilution of an AIDS patient serum containing high titers of anti-HIV antibodies (Ab) recognizing most viral proteins (32). Filters were then washed and incubated for 90 min with [^{125}I]-labeled protein A (200,000 dpm/ml), washed again, air-dried, and exposed overnight to X-ray film.

Northern Blot for HIV-1 and Monocyte Chemoattractant Protein-1 (MCP-1) RNAs

U1 cells were either left unstimulated or were stimulated with TNF-α or IL-6 in the presence or absence of Dex for 48 or 5 h for HIV-1 and MCP-1, respectively. RNA extraction was performed using an RNA isolation kit (Stratagene, La Jolla, CA). Northern blot analysis was performed as previously described (32,34). Briefly, 10 μg of each sample was electrophoresed through a 0.8% formaldehyde agarose gel. The RNA was transferred to nitrocellulose by Northern blotting and filters were hybridized with an [α-^{32}P]-labeled homologous HIV LTR probe (Sst I-BssHII) and then stripped and re-hybridized with a labeled [α-^{32}P]-labeled-β-actin probe. For MCP-1 RNA detection human MCP-1 cDNA (0.672 Kb PstI-PstI fragment) (41) was labeled by the "ready to go" DNA labeling system (Pharmacia Upjohn) with [α^{32}P]-dCTP (3,000 Ci/mmol, Amersham). The human glucocorticoid receptor cDNA probe was kindly provided by Dr. Ronald Evans (The Salk Institute for Biological Studies, La Jolla, CA), whereas the human IL-6 receptor cDNA probe was kindly provided by Dr. Tadamitsu Kishimoto (Osaka University, Osaka, Japan).

Electrophoretic Mobility Shift Assay (EMSA) for Activating Protein-1 (AP-1)

AP-1 analysis was performed on nuclear cell extracts prepared from 5×10^6 of pelletted U1 cells, resuspended and lysed on ice in 25 μl of buffer A containing 0.2% of NP-40 for 10 min and vortexed for 10 seconds. Buffer A (10mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT) was supplemented with the following protease inhibitors: leuptin (10 μg/mL), pepstatin A (10 μg/mL), aprotinin (33μg/mL), E-64 (10μg/mL), AEBSF (1 mmol/L), diisopropyl fluorophospate (DFP) (3 mmol/L), and with the following phosphatase inhibitors: sodium vanadate (Na_3VO_4) (1 mol/L), sodium fluoride (NaF) (50 mmol/L). After centrifugation, the supernatant was removed and the pelleted nuclei were resuspended in 20 μl of cold buffer C (20 mM HEPES, pH 8.0; 25% glycerol; 0.42 M NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 0.5 mM DTT, plus the above listed protease and phosphatase inhibitors) for 15 min on ice. After centrifugation, supernatant was collected, analyzed for protein concentration and tested for EMSA. Equal amounts of nuclear cell extracts (2 μg) were added to a reaction mixture composed of binding buffer (20 mM HEPES, pH 8.0; 20% glycerol; 100 mM KCl; 0.6 mM EDTA; 5 mM DTT; 100 mM KCl, 1mM $MgCl_2$, 0.3 mM PMSF, 4 μg BSA), 1 μl of poly(dI:dC, 5 mg/ml) (Pharmacia Upjohn) and 5×10^4 cpm of labeled AP-1 probe: 5′-CGC TTG ATG AGT CAG CCG GAA-3′, 3′-GCG AAC TAC TCA GTC GGC CTT-5′ (Promega, Madison, WI) labeled with [^{32}P]-ATP (Amersham) using polynucleotide kinase T4 (New England Biolabs, Beverly, MA) in a final volume of 15 μl and incubated for 30 min at room temperature (rt°). For supershifting experiments, parallel aliquots of nuclear cell extracts were incubated in the presence or absence of 1 μg of Ab (Santa Cruz Biotecnology, Inc., Santa Cruz, CA) directed to different components of AP-1 for 30 min at rt°. One μl of [^{32}P]-ATP-labeled double-stranded probe (0.5 ng) was then added and the reaction was incubated at rt° for 30 min and then run on a 5% acrylamide gel in 1× TBE (0.0045 M Tris-borate, 0.001 M EDTA) for 4 h at 150 V. Radioactive bands were revealed by autoradiography of dried gels.

Western Blot Analysis of ERK1/2 Mitogen-activated Protein Kinases (MAPK)

Ten μl of nuclear cell extracts were electrophoresed on a 10% SDS-PAGE and subsequently transferred to a nitrocellulose membrane (Hybond ECL, Amersham, Little Chalfont, UK) by electroblotting. Membranes were incubated with a blocking solution of 5% nonfat milk, 20 mM Tris, pH 7.6, 137 mM NaCl, 0.2% Tween 20, for 1 h at rt°, and further incubated overnight at 4°C with monoclonal Ab (mAb) recognizing phoshorylated-ERK, D-4 (Santa Cruz Biotechnology). mAb binding was visualized by using horseradish peroxidase-conjugated anti-mouse Abs (Amersham Pharmacia Biotech). The signal was revealed by the enhanced chemiluminescence system (ECL, Amersham) according to the manufacturer's instructions. For determination of the total amount of ERK1/2, the filters were stripped and re-blotted using the anti-ERK2 mAb D-2 (Santa Cruz Biotechnology). Biotinylated protein markers were purchased from Cell Signaling Technology (Beverly, MA).

LTR-Chloramphenicol Acetyl Transferase (CAT) Transient Transfections

10^7 U1 cells were transiently transfected with an HIV-1 LTR CAT construct (one μg of plasmid DNA per $5-10 \times 10^6$ cells) by hypotonic DEAE dextran, as described (32). Four h after transfection, cells were stimulated as indicated and protein extracts were prepared 36 h after transfection. Cell supernatants were harvested from the remainder of the culture 72 h after transfection and stored at −80°C for determination of the RT activity content. For the

generation of protein extracts, U1 cells were washed in phosphate-buffered saline (PBS), resuspended in 100 μl of Tris (100 mM, pH 7.5) plus 0.1% Nonindet P-40 (NP-40), and disrupted by three cycles of freezing and thawing. The cell debris was pelleted and the protein concentration of the supernatant was measured with a protein assay kit (Bio-Rad, Richmond, CA). Each CAT assay was adjusted to contain equal amounts of protein from each individual transfection. Fold induction of CAT activity was calculated by dividing the counts per min (cpm) of the test samples by the cpm of the samples obtained from unstimulated cells. In some experiments, uninfected parental U937 cells (41) were transfected and stimulated by cytokines and/or Dex following the same procedure.

Results

Synergistic Induction of HIV Expression in U1 Cells by Stimulation with IL-6 and GC

The U1 cell line is a well-characterized model of inducible HIV-1 expression (38). Northern blot analysis indicated that U1 cells constitutively express 2 Kb mRNA in the absence of detectable levels of unspliced 9 Kb transcripts suggestive of a Rev-dependent latency (37). Subsequent studies have, however, linked the phenotype of restricted virus expression of U1 cells to a defective function of Tat rather than Rev (42–44).

Expression of the GC receptor in U1 cells was observed by Northern blot analysis using a cDNA probe for the human GC receptor (data not shown). In addition, receptor binding studies using [^{3}H]-Dex indicated that unstimulated U1 cells express approximately 3,800 receptors per cell (Table 1), a number of molecules adequate to transduce biological effects (40). Stimulation of U1 cells with Dex alone did not induce HIV expression from U1 cells, as measured by supernatant-associated RT activity, whereas IL-6 upregulated virus production (34). As observed with TNF-α (32) co-stimulation of U1 cells with IL-6 and Dex resulted in a synergistic effect on virus production lasting for several days of culture (Figure 1A). The synergistic effect observed in U1 cells co-stimulated by cytokines and Dex was mediated by a functional intracellular receptor, as demonstrated by the ability of the receptor antagonist RU 486 (39) of suppressing the effect of GC on cytokine-mediated HIV expression in these cells (Figure 1B). In addition to increase RT activity, Dex enhanced the accumulation of cell-associated HIV proteins induced by IL-6, as demonstrated by Western blot analysis (Figure 2).

The steroid potentiating effect was relatively specific in that virus expression following U1 cell stimulation with granulocyte-macrophage colony stimulating factor, another HIV-inductive cytokine (35,45,46) was not further enhanced by Dex (data not shown). No toxicity or substantial changes in the levels of cell proliferation were observed in the different experimental conditions tested. No further increase of RT activity was seen when cells were stimulated with higher concentrations (up to 10^{-5} M) of Dex in the presence or absence of IL-6 (data not shown).

Since GC have been reported to upregulate IL-6 receptor mRNA and cell surface expression in epithelial and epathoma cell lines (27,47) we have investigated whether the observed synergy could be accounted for by a similar effect in U1 cells. Total RNA was isolated from U1 cells after 8 and 24 h of stimulation with IL-6, Dex or their combination, and probed for IL-6 receptor. However, Dex did not increase the accumulation of IL-6 receptor message at either time point. Furthermore, IL-6 stimulation did not upregulate the number of GC receptors in U1 cells, as determined by Northern blot and/or binding studies (data not shown).

Lack of Effect of IL-6 and Dex on Steady-state HIV RNA Accumulation

The predominant effect of IL-6 in U1 cells is a post-transcriptional activation of HIV expression occurring without evident accumulation of viral transcripts, as determined by Northern blot analysis (34). Consistently with the lack of effect on both released RT activity and expression of cell-associated viral proteins, U1 cell stimulation with Dex alone did not increase the low constitutive levels of 2 Kb mRNA. IL-6 did not upregulate HIV transcripts either in the presence or absence of Dex (Figure 3A) whereas the steroid potentiated TNF-α mediated upregulation of viral RNA, as previously reported (32). Therefore, Dex-mediated

Table 1. Specific binding of GC in U1 cells

Total Binding (cpm)	Nonspecific Binding (cpm)	Specific Binding (cpm)	Receptors Per Cell
1,081 ± 28	424 ± 15	657 ± 17	3,800 ± 98

Duplicate samples of 5×10^6 U1 cells were labeled in the presence of [^{3}H]-Dex (total binding) or [^{3}H]-Dex plus an excess of unlabeled steroid (nonspecific binding). Specific binding to GC receptors was determined by subtracting the nonspecific binding value from that of the total binding, whereas the number of receptors per cell (±S.E.M.) was determined from the specific activity of the labeled Dex.

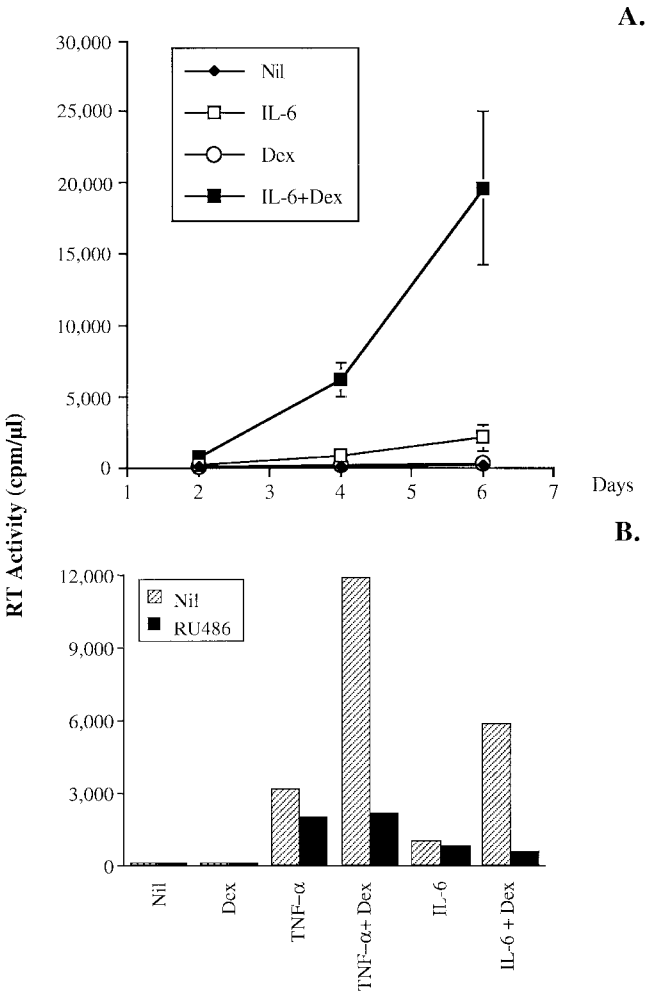

Fig. 1. Synergistic induction of HIV expression by IL-6 and Dex in U1 cells. (A) Kinetics of RT activity produced in culture supernatants after an individual stimulation with IL-6, Dex, or co-stimulation with both agents. The results show the mean ± SD of 10 independent experiments. **(B)** RU 486 blocks the synergistic induction of HIV expression in U1 cells co-stimulated with Dex and cytokines. The results represent peak RT activities measured 4 days after treatment, and were confirmed by determinations performed at day 6. In some experiments, hydrocortisone instead of Dex was used in the same range of concentrations with identical results.

potentiation of IL-6 induced virus production was unlikely accounted for by substantially increased levels of HIV transcription and/or HIV RNA accumulation, at least by Northern blot analysis.

Dex Inhibits IL-6 Mediated Induction of MCP-1 RNA

We have previously reported that IL-6 stimulation of both uninfected U937 cells and of the U1 cell line induced the CC-chemokine MCP-1, both at the RNA and protein levels (41). Dex stimulation inhibited the accumulation of MCP-1 mRNA induced by IL-6 in U1 cells (Figure 3B), indicating that the lack of synergistic effect with IL-6 on the accumulation of HIV-1

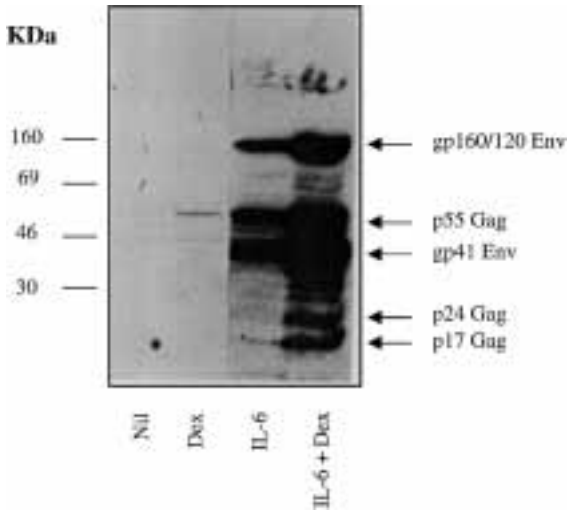

Fig. 2. Western blot analysis of cell-associated HIV-1 proteins. Lysates were prepared from U1 cells either unstimulated or stimulated for 48 h with Dex (10^{-8} M), IL-6 (100 U/ml), or co-stimulated with IL-6 and Dex. The molecular weight markers are indicated on the left and the corresponding major HIV proteins on the right.

transcripts was not a reflection of a generalized refractivity of U1 cells to modulatory effects of GC on gene transcription.

Dex Accelerates AP-1 and ERK1/2 MAPK Activation in U1 Cells Stimulated with IL-6

In addition to NF-kB dependent transcription, AP-1 activation has been recently shown to mediate cytokine-induced upregulation of virus expression in different cells, including U1 cells stimulated with IL-6 (48). Therefore, we investigated whether Dex influenced formation of an active AP-1 complex either alone or in the presence of IL-6. Constitutive AP-1 binding was present in nuclear extracts prepared from unstimulated U1 cells. IL-6 induced AP-1 binding in U1 cells after 20 h of stimulation, as reported (48), but not earlier. Dex alone did not modify the baseline pattern of AP-1 binding, that, however, was enhanced after 8h of co-stimulation by IL-6 plus Dex, indicating that the steroid hormone accelerated IL-6 induced AP-1 binding (Figure 4A). Ab-mediated supershift analysis of the Ap-1 complex composition induced by IL-6 plus Dex stimulation of U1 cells revealed the presence of JunD, c-Fos, Fra-1 and Fra-2 transcription factors (Fig. 4B). The increased levels of AP-1 activation observed after 8 h of co-stimulation by IL-6 and Dex reflected an analogous pattern of ERK1/2 phosphorylation, that was no longer detectable 20 h post-stimulation (Fig. 4C).

These results indicate that IL-6 and Dex can synergize in the induction of certain transcription factors in U1 cells, although this inductive effect does not appear sufficient to increase HIV transcription, as shown in Fig. 3A.

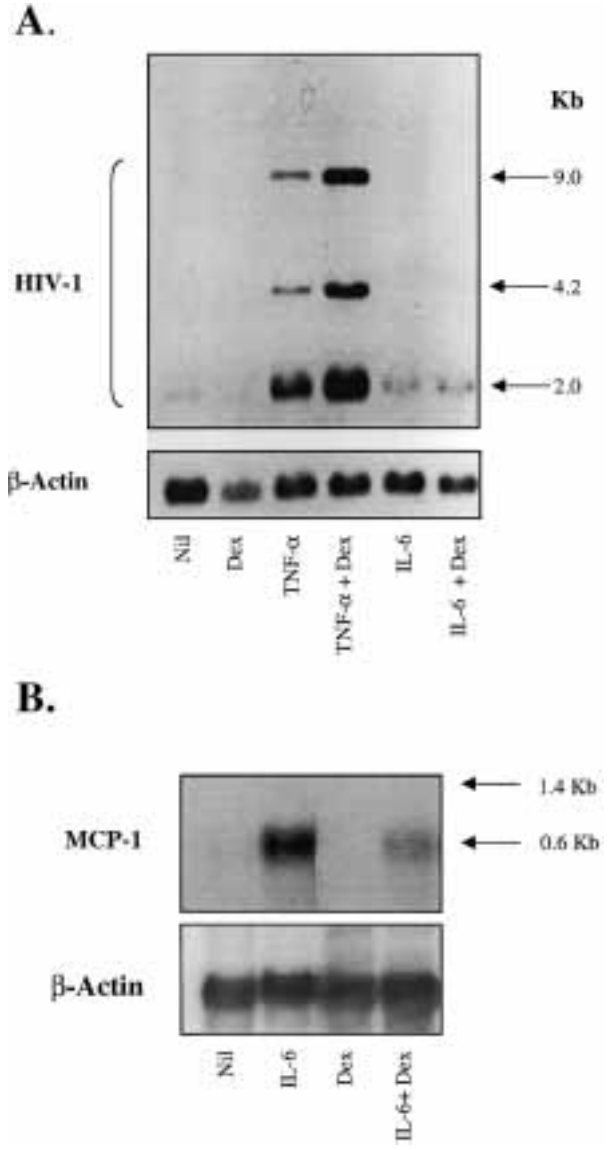

Fig. 3. Northern blot analysis of HIV-1 and MCP-1 RNA. Total RNA was extracted from unstimulated and stimulated U1 cells after 48 h of culture for HIV RNA, and after 5 h of culture for MCP-1. **(A)** As reported (32), Dex upregulated the expression of HIV RNA in U1 cells following cell incubation with TNF-α, whereas it did not alter the low levels of transcripts observed in this cell line either left unstimulated or stimulated with IL-6. **(B)** IL-6 activates MCP-1 expression in U1 cells (41) and this effect is inhibited by Dex.

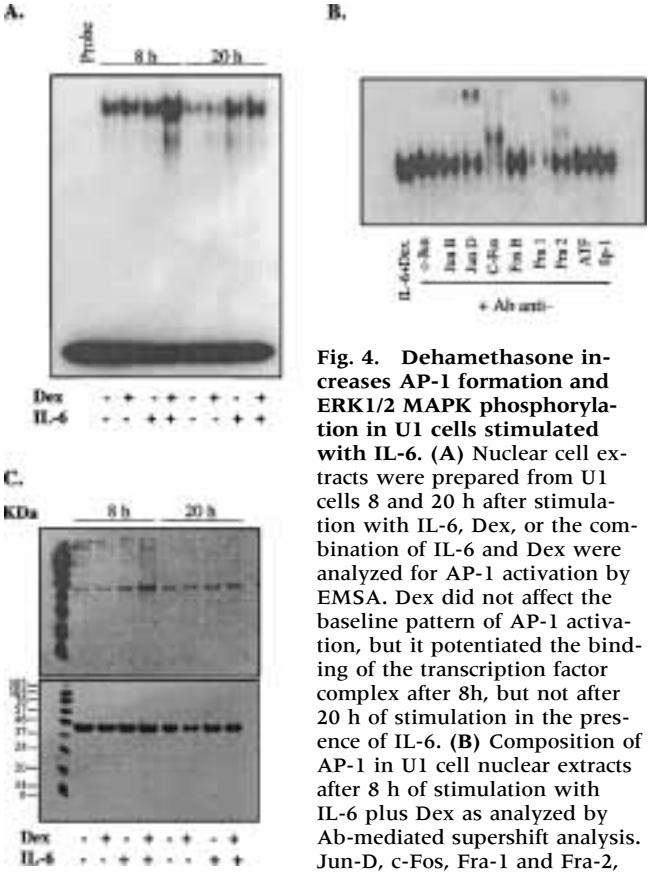

Fig. 4. Dehamethasone increases AP-1 formation and ERK1/2 MAPK phosphorylation in U1 cells stimulated with IL-6. (A) Nuclear cell extracts were prepared from U1 cells 8 and 20 h after stimulation with IL-6, Dex, or the combination of IL-6 and Dex were analyzed for AP-1 activation by EMSA. Dex did not affect the baseline pattern of AP-1 activation, but it potentiated the binding of the transcription factor complex after 8h, but not after 20 h of stimulation in the presence of IL-6. **(B)** Composition of AP-1 in U1 cell nuclear extracts after 8 h of stimulation with IL-6 plus Dex as analyzed by Ab-mediated supershift analysis. Jun-D, c-Fos, Fra-1 and Fra-2, unlike the other transcription factors examined, were supershifted by their respective Abs; the anti-Sp1 Ab was used as negative control. **(C)** ERK1/2 activation (upper panel) and total amount of ERK1/2 (bottom panel) were measured in the same nuclear extracts of U1 cells analyzed for AP-1 activation. Molecular weight markers are shown on the left side. These results are representative of those obtained in three independent experiments.

IL-6 and Dex Stimulate HIV Expression in U1 and U937 Cells Independently from the Activation of the Virus LTR

The results obtained thus far suggested that Dex and/or IL-6 did not significantly affect transcription and the steady-state accumulation of viral transcripts above the constitutive levels typical of unstimulated U1 cells. U1 cells were next transiently transfected with an HIV-1 LTR-CAT plasmid, as described (32), and were either left unstimulated or were stimulated for 4 to 6 h with Dex, TNF-α, IL-6, or combinations of the individual cytokines and of the steroid hormone. Activation of both the heterologous HIV-LTR plasmid, measured by CAT activity, and of the endogenous proviral DNA, as measured by the production of RT activity, were observed when transfected U1 cells were stimulated with TNF-α (Figure 5A). Of note is the fact that both RT and CAT activities were upregulated by TNF-α at concentrations as low as 1 U/ml, indicating a comparable sensitivity of the two assays in this model system. No effects were observed in terms of either CAT or RT activities in U1 cells stimulated with Dex alone; however, TNF-α induced activation of LTR-driven CAT activity and Dex potentiated this effect (Figure 5B), as previously reported (32). In sharp contrast, negligible activation of CAT activity

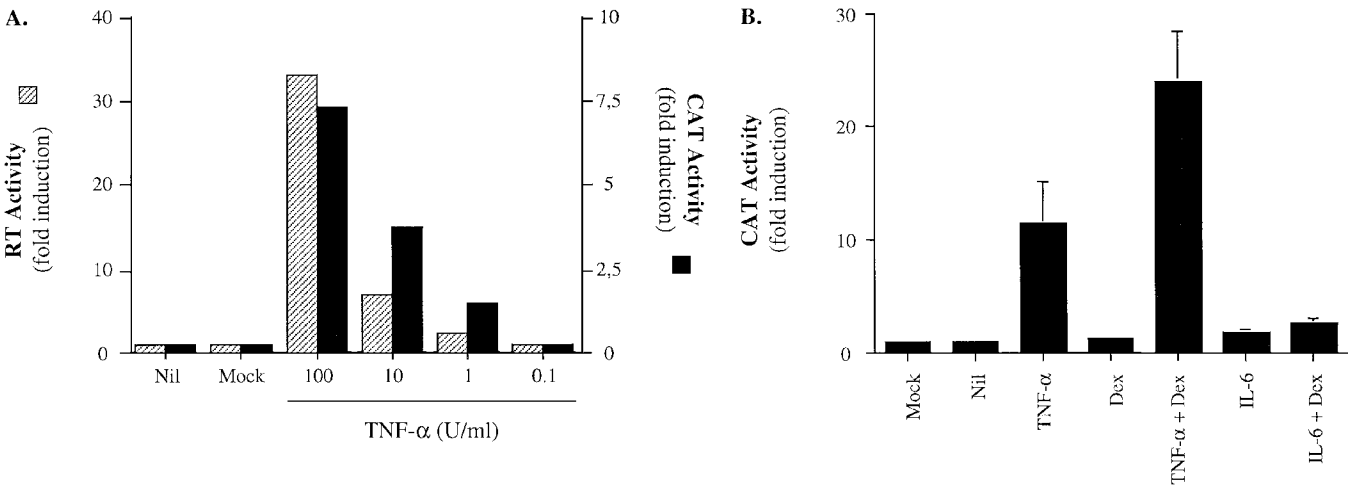

Fig. 5. LTR-independent synergistic induction of HIV expression in U1 cells by IL-6 and Dex. (A) Activation of supernatant-associated RT and CAT activities in U1 cells transiently transfected with an HIV-1 LTR-CAT construct and stimulated with different concentrations of TNF-α 48 h after transfection. Although quantitatively different, the sensitivity of U1 cells to the activating effect of TNF-α was comparable in both assays. **(B)** Lack of HIV LTR activation by stimulation of U1 cells with IL-6, in the presence or absence of Dex. The results represent the mean ± SD of 4 independent experiments. In no experiments did IL-6 alone, Dex alone, or their combination induce CAT activity at levels above 2-fold the low constitutive levels observed in unstimulated U1 cells.

was observed when transfected U1 cells were stimulated with IL-6 alone, or with IL-6 plus Dex (Figure 5B). These results strongly suggest that the induction of HIV expression observed in U1 cells co-stimulated with IL-6 and Dex does not involve the upregulation of LTR-driven transcription. In support of this interpretation, stimulation of U1 cells with IL-6 alone (49) or in the presence of Dex did not activate the cellular transcription factor NF-kB (data not shown).

In order to rule out the possibility that the observed effects were peculiar of U1 cells, uninfected U937 cells were transfected with the HIV-1 LTR-CAT plasmid and then stimulated with either TNF-α or IL-6 in the presence or absence of Dex. No activation of the virus LTR was observed in U937 cells in the presence of IL-6 or IL-6 plus Dex in contrast to TNF-α that, either alone or in the presence of Dex, induced CAT activity to an extent comparable to that observed in U1 cells (data not shown).

Discussion

In the present study, we have demonstrated that a synergistic induction of HIV-1 expression is triggered in chronically infected U1 cells simultaneously stimulated with IL-6 and Dex. Co-stimulation of U1 cells with the two agents potently induced viral protein expression and *de novo* virion production independently from either HIV LTR activation or evident accumulation of steady-state HIV transcripts (Fig. 6).

GC mediate their biological effects by binding to cytoplasmic receptors followed by translocation of the steroid/receptor complex to the cell nucleus where recognition of specific target sequences occurs (50,51). Transcriptional activation occurs after interaction of the «zinc finger» DNA binding domain of the glucocorticoid receptor to a DNA sequence represented by the perfect palindrome AGAAACAnnnTGTTCT. Since minimal variations of this sequence are present in the enhancers of different genes, a consensus GC response element (GRE) is represented by the sequence GGTACAnnnTGTTCT (52). In addition, GC receptors can interact with several transcription factors including Jun-Fos (AP-1) (53,54) among others (55).

In addition to cellular genes, GC control the transcription of animal retroviruses including murine mammary tumor virus (50,56) and Moloney murine sarcoma virus (57). Concerning HIV, it has been reported that Vpr, an accessory gene inducing cell-cycle arrest, can functionally interact with the glucocorticoid receptor resulting in the potentiation of virus replication (58–61) and HIV expression from different chronically infected cell lines, including U1 (62). Since this effect has been linked to the activation of NF-kB (63), it is unlikely that the potentiating effects here described in U1 cells co-stimulated with IL-6 and Dex could be ascribed to a similar mechanism. In this regard, the HIV-1 genome contains multiple potential GRE throughout its sequence, with the strongest homology likely occurring in the portion of U3 LTR known as «negative regulatory element» at positions 190–204 in the HXB-2 reference sequence (Genbank ascension number K03455). However, deletion analyses have failed to assign a functional role to this portion of DNA (11–13). Indeed, we could not demonstrate an increase of GC receptor mediated transcription of

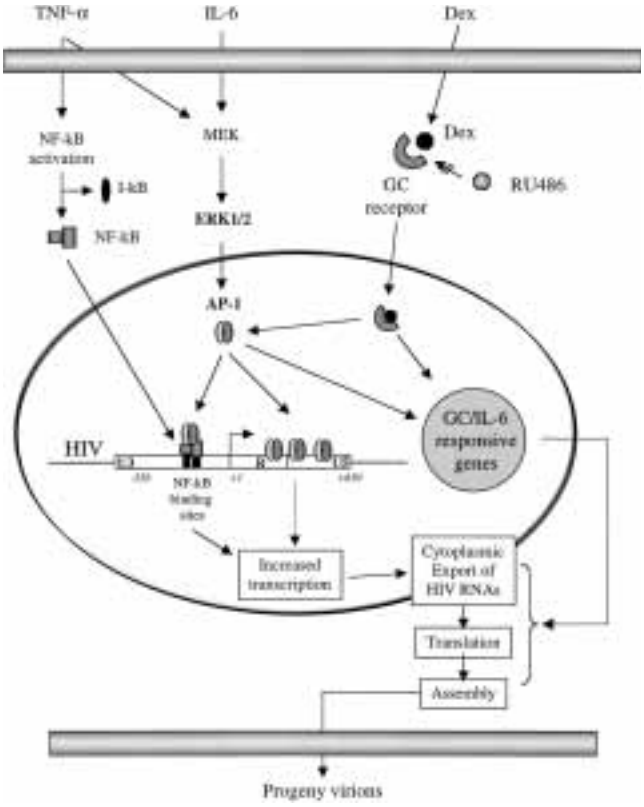

Fig. 6. Model of transcriptional and post-transcriptional synergistic induction of HIV expression in U1 cells by cytokines and GC. IL-6 induces activation of ERK1/2 MAPK and AP-1 that can lead to increased viral transcription if U1 cells are simultaneously stimulated with TNF-α, a strong inducer of NF-kB activation. Direct binding of AP-1 complexes to HIV DNA sequences downstream the transcription start site may not always result in increased transcription. Ultimate post-transcriptional synergy can occur in U1 cells co-stimulated by IL-6 and GC as a result of the activation of target cellular and viral genes.

HIV following cell stimulation with steroids by nuclear run-on analysis, or, as here presented, in U937 or U1 cells transiently transfected with an HIV LTR CAT plasmid and stimulated with Dex alone or together with IL-6.

GC biological effects are, however, not limited to transcriptional control of target genes, and their capacity to affect protein expression by acting on different post-transcriptional mechanisms has been well documented. Examples include the suppression of globin mRNA translation in Friend leukemia cells undergoing differentiation (64), the inhibition of α1-acid glycoprotein RNA (65), the accumulation of growth hormone RNA (66), and the inhibition of pro-inflammatory cytokines such as IL-1β and TNF-α synthesis (19–21). The observation here reported that RU486 blocks the ability of GC to synergize with both TNF-α (32) and IL-6 in the induction of HIV expression indicates that GC receptors are involved in mediating both transcriptional and post-transcriptional effects in U1 cells.

GC have been found to exert both transcriptional and post-transcriptional effects on the IL-6 axis in different model systems (67–72). In a previous study, GC was described to increase the number of IL-6 receptors (27), thus providing a potential explanation for the synergistic effect observed in our study that, however, was not observed in U1 cells. Furthermore, protein-protein interaction between the GC receptor and NF-IL-6 were reported to affect the levels of rat α1-glycoprotein expression (29). However, in the present study, no evidence of cross-modulation of either IL-6 or GC receptors after cell stimulation with IL-6 and Dex was obtained by Northern blotting and by direct binding assay for GC.

The precise mechanism of induction of HIV expression by IL-6 and Dex in U1 cells remains undefined. Both agents display the intrinsic capacity to potentiate HIV transcription in both U1 cells and other model systems in the presence of other stimuli such as TNF-α (32,34) or Tat (48). In this regard, it has been recently proposed that several cytokines, including IL-6, can induce AP-1 via activation of ERK1/2 MAPK leading to formation of AP-1 complexes that cooperated with NF-kB in driving viral transcription from the LTR in U1 and U937 cells co-stimulated by different cytokines (48). In the same study, however, the authors confirmed our original observation that IL-6 stimulation *per se* does not lead to substantial NF-kB activation or viral transcription in U1 cells, although it potentiated TNF-α induced HIV transcription and virion expression (34,49). An alternative possibility is that increased viral transcription may occur by activation of an intragenic enhancer containing three functional AP-1 binding sites (73–75). In support of this hypothesis, Dex enhanced both ERK1/2 activation and the formation of AP-1 complexes induced by IL-6 after 8 h, but not after 20 h of stimulation. Although GC usually inhibit AP-1 formation as part of their general anti-inflammatory mechanism by the so called "AP-1 GC receptor transcriptional cross-talk" (76,77) potentiation has also been reported (78). Furthermore, Dex-mediated enhancement of IL-6 signaling also been reported in Kaposi's sarcoma cells (31). Either the transient nature of the synergy or the composition of the AP-1 complexes, however, were not sufficient to induce evident accumulation of viral transcripts in contrast to what observed with U1 cells co-stimulated with TNF-α and Dex. In this regard, uncoupling of DNA binding from transcriptional enhancement by Ap-1 has been previously documented (79,80). The observation that levels of HIV expression measured by supernatant-associated RT activity, a faithful indicator of virion production (81), comparable to those induced by TNF-α can be achieved without evident changes in terms of cell-associated viral transcripts strongly argues in favor of a predominant post-transcriptional effect triggered by IL-6 and GC stimulation of U1 cells. In this regard, similar observation of GC-mediated

effect independent from the enhancer region itself has also been previously reported in CEM-T4 cells stimulated by PMA, TNF-α or Tat (82). Among other potential mechanisms, activation of ERK1/2 MAPK has been shown to affect HIV-1 infectivity during acute viral replication (83), a mechanism that has been linked to phosphorylation of the *vif* gene product (84,85).

Our current study implies that even a profound inhibition of HIV-1 gene transcription may not ultimately result in a total impairment of HIV replication as long as cytokines such as IL-6 and GC are present. Worthy of note, upregulated levels of IL-6 have been reported in HIV-infected individuals (86–88). These findings are also of relevance in consideration of the broad clinical use of GC in AIDS-related disorders (89) where they are utilized as anti-inflammatory agents (90–93).

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