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A quantitative bioassay for HIV-1 gene expression based on UV activation: effect of glycyrrhizic acid

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Abstract

Previous reports have shown that HIV-LTR*cat* constructs stably transfected in HeLa cells are inducible after exposure to UV light. We have optimized this system for studying the effect of drugs on HIV-1 gene expression. The maximum UV response was observed in quiescent stationary cells stimulated with fresh medium for 3 h. Glycyrrhizic acid suppressed UV-induced HIV gene expression in a concentration-dependent manner. The inhibitory effect was strongest when GL was added immediately after UV exposure; it was still evident when GL was added at 5 h, it was completely lost at 10 h, after UV exposure. The inhibitory effect was even more pronounced if the cells were pretreated with sub-effective dose (0.0012 mM) of GL prior to UV exposure. The IC_{50} values with and without pretreatment were 0.04 and 0.38 mM, respectively. Cell proliferation and viability were not affected by GL at doses as high as 2.4 mM. The inhibitory effect of GL on UV-induced CAT activity correlated with the complete inhibition of binding activities of NF-κB p65, NF-κB p50, c-Fos, and c-Rel. Thus, the UV-based bioassay as proposed here can be exploited for the routine screening of the compounds that interfere with HIV-1 gene expression.

Keywords: Glycyrrhizic acid; HIV-1 gene expression; UV activation

1. Introduction

Licorice extract is a herbal drug, which has long been used as a demulcent and elixir in Chinese medicine. Glycyrrhizic acid (GL), an active component of licorice roots (*Glycyrrhizae radix*), has been shown to be active against a variety of viruses including herpes simplex type 1 (HSV-1), varicella-zoster virus (VZV), human cytomegalovirus (HCMV), hepatitis A, B, and C (HAV, HBV, and HCV) viruses, influenza virus, and human immunodeficiency virus-1 (HIV-1) (Pompei et al., 1979; Baba and Shigeta, 1987; Numazaki et al., 1994; Crance et al., 1994; Sato et al., 1996; Arase et al., 1997; Ito et al., 1987, 1988; Utsunomiya et al., 1997). Recently it has been reported from this laboratory that GL also inhibits Epstein-Barr virus (EBV) replication in vitro (Lin, 2003). In addition, GL has been extensively studied in relation to various biological effects, including anti-inflammatory activity (Finney and Somers, 1958), interferon-inducing ability (Shinada et al., 1986; Abe et al., 1982). Clinically, GL has been used to treat patients with chronic active hepatitis (Suzuki et al., 1983).

Previous reports have shown that HIV-1 gene expression, assayed by LTR-directed expression of the chloramphenicol acetyltransferase (CAT), can be induced by UV irradiation (Valerie et al., 1988; Valerie et al., 1995a,b). However, the level of induction varied in each experiment resulting from the variation in the basal level of activity. In this study we optimized the UV induction system and exploited this bioassay system for studying the effect of drugs on HIV-1 gene expression.

In view of the broad spectrum of antiviral activities and pharmacological effects exerted by GL, we decided to investigate its effect on the UV-induced expression of CAT

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transcriptionally driven by the HIV-LTR promoter. The results clearly indicate that GL suppresses UV-induced HIV-1 gene expression in a concentration-dependent fashion.

2. Materials and methods

2.1. Cells and culture conditions

The stably transfected HeLa HIV-LTR*cat* cells, harboring the bacterial CAT gene under control of the HIV-LTR (−485/+80), were generously provided by Valerie et al. (1988). All cell cultures were maintained in DMEM containing 4500 mg/l D-glucose–10% fetal bovine serum (FBS) supplemented with penicillin, streptomycin, and geneticin (50 μg/ml). Cultures were incubated at 37 °C in a CO_2 (5%) incubator. Cell viability was determined by trypan blue exclusion.

2.2. UV irradiation

All UV irradiations were carried out using a 254-nm germicidal lamp in a Stratalinker UV Crosslinker chamber (Stratagene, Inc., La Jolla, CA, USA). The cells were covered with medium during UV irradiation.

2.3. Drug treatments

Glycyrrhizic acid (GL) (Acros Organics, New Jersey, PA, USA) was dissolved in PBS, pH 7.5. The quiescent cells were stimulated with fresh medium containing 10% FBS for 3 h prior to UV irradiation. Cells were treated with various concentrations (0.024–2.4 mM) of GL immediately after UV exposure. Cells were also treated with 3 μM trichostatin A (TSA) (Calbiochem-Novabiochem Corporation, La Jolla, CA, USA), or 50 mM sodium butyrate (SB) (Sigma, St. Louis, MO, USA), or 0.5 mM H_2O_2. After 24 h of treatment with these drugs, cells were harvested for performing the CAT assays.

2.4. CAT assays

The cells were washed twice with cold 0.25 M Tris–HCl, pH 7.5, scraped, and harvested in the same buffer. The cell extracts were prepared by three cycles of freeze–thawing and protein concentrations were determined by the method of Bradford (BioRad). CAT activity was determined essentially as described (Gorman et al., 1982) using samples normalized for protein content. A reaction mix of 150 μl containing 20 mM acetyl CoA (Sigma Chemical Co.), 0.3 μCi [^{14}C]chloramphenicol (Amersham), and 100 μg of protein was incubated for 1 h at 37 °C. Reaction products were analyzed and quantitated by the Phosphor Imager Analyzer.

2.5. Detection of cellular transcription factors

To detect DNA binding by specific transcription factors associated with the UV-induced HIV-1 gene expression, an enzyme-linked immunosorbent assay (ELISA)-based format was used (Clontech Laboratories, Inc.). This method is faster, easier, and more sensitive than electrophoretic mobility shift assays and does not require the use of radioactivity. The assays were performed according to the Manufacturer's protocol.

Nuclear extracts were prepared as previously described (Andrews and Faller, 1991).

3. Results

3.1. Optimization of UV induction

Previous reports indicate that HIV-LTR*cat* constructs in stably transfected HeLa cells are inducible after exposure to different types of DNA-damaging agents, including UV (Valerie et al., 1988, 1995b) and *cis*-platinum (Woloschak et al., 1995). However, the level of induction varied in each experiment resulting from the variation in the basal level of activity. Our initial experiments were designed to study the effects of cell growth on basal level expression of HIV. Fig. 1 clearly shows that the expression of HIV as measured by CAT activity fluctuated during the incubation period. At 10 h after subculture, HIV expression was low, increasing with time and reaching a peak at 40 h when cells were at the phase of exponential growth. As the cells became confluent (day 3) and entered into a quiescent state (after day 4), the HIV expression decreased to its lowest level (day 7). Upon exposure of these quiescent cells (day 4) to UV irradiation, CAT activity was only increased approximately two-fold of the exponentially growing cells (40 h after subculture) and seven-fold of the quiescent cells. These results demonstrate that the basal level of CAT activity fluctuates with the physiological condition of the cells, which, in turn, may affect the magnitude of UV activation.

To determine the conditions for maximal UV induction of HIV expression, the quiescent stationary cells (day 7) were stimulated with fresh medium and at different times cells were exposed to UV induction. As shown in Fig. 2, UV induction of CAT activity was maximal at 3 h after changing the medium, producing approximately 30-fold increase relative to quiescent cells without UV exposure and CAT activity gradually decreased with time thereafter. These results indicate that the physiological condition of the cells at the time of UV exposure might be one of the critical determinants of the magnitude of response. Thus, to normalize the basal level of expression and increase the sensitivity of UV activation for subsequent experiments, cells were first grown for 7 days without additional medium followed by stimulation with fresh medium for 3 h before UV irradiation. This system not only produced consistent and reproducible

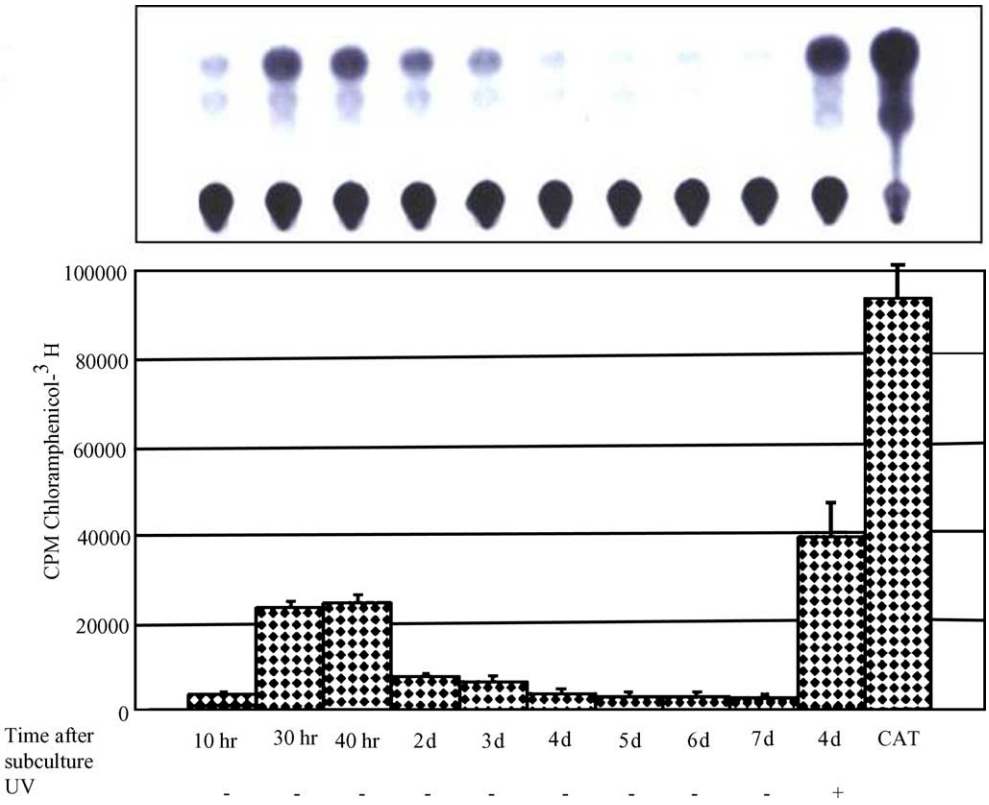

Fig. 1. Effects of cell growth on constitutive HIV expression. The stably transfected HeLa HIV-LTR*cat* cells were maintained in DMEM plus 10% FBS. At different time points after subculture, aliquots of the cells were taken for CAT assay. Reaction products were analyzed by thin layer chromatography and were quantitated by Phosphor Imager Analyzer. The basal level of CAT activity reached a peak between 32 and 40 h after subculture when cells were at the phase of exponential growth. Upper panel, a representative radioactive images; lower panel, average results of three separate experiments with standard deviation bars; CAT, the bacterial chloramphenical acetyltransferase as a positive control. Unless otherwise stated, all results in the subsequent experiments were obtained from the average of three separate experiments with the standard deviation bars.

results of UV activation but also allowed accurate estimation of the GL dose effects on HIV-LTR*cat* activity.

3.2. Determination of optimal UV doses

The quiescent HeLa HIV- LTR*cat* cells were stimulated with fresh medium containing 10% FBS for 3 h and irradiated with various doses of UV light. The CAT activity was increased as a function of the UV dose (data not shown). The induction of CAT activity was evident at 6 mJ/cm^2 and leveled off between 8 and 12 mJ/cm^2. Concomitantly with the increase in CAT activity, there was no significant increase in the percentage of cell death up to 12 mJ/cm^2. Cell viability was maintained at 89–94% within the dose range tested. Further increases in UV dose up to 20 mJ/cm^2 resulted in a decrease of CAT activity and increased cell killing up to 35% (data not shown).

To determine the kinetics of CAT expression, we measured the levels of CAT activity at various times after UV irradiation at a dose of 10 mJ/cm^2. CAT activity could first be detected between 2 and 4 h, reaching maximum levels between 20 and 24 h, and then decreasing from 24 to 48 h after irradiation (data not shown). Thus, all subsequent experiments, unless otherwise stated, were carried out with the quiescent cells stimulated for 3 h, irradiated with the UV dose of 10 mJ/cm^2, and assayed for CAT activity 24 h after UV exposure.

3.3. Effects of GL concentration on UV-induced HIV expression

To examine the effects of GL on UV-induced expression of HIV-LTR*cat*, cells were irradiated with 10 mJ/cm^2 and then immediately treated with various concentrations of GL for 24 h before harvest. As shown in Fig. 3, the GL-mediated inhibition of UV-induced HIV expression was GL concentration-dependent. In the presence of 0.024 mM of GL, HIV-dependent CAT activity was slightly decreased, but the activity was further decreased with increasing concentrations of GL. At a concentration of 1.2 mM, the level of CAT activity was reduced to the basal level of the untreated control. It should be noted that various concentrations (0.024–2.4 mM) of GL administered alone demonstrated little effect on basal level of HIV expression (data not shown).

Additional experiments were performed to examine the effects of varying the concentration of GL on cell proliferation and viability. In these experiments, cells were treated with various concentrations of GL at the time of subculture,

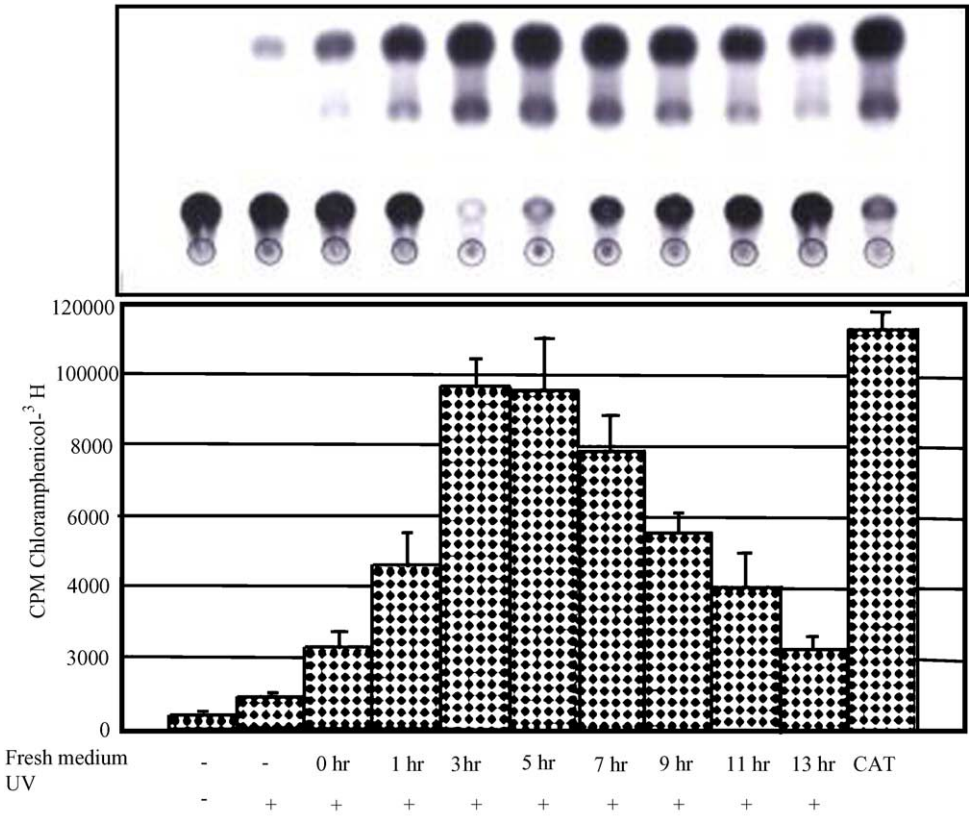

Fig. 2. Effects of cell growth on the UV-induced HIV expression. The stably transfected HeLa HIV-LTR*cat* cells were grown for 7 days and then stimulated with fresh medium containing 10% FBS. At different times after fresh medium stimulation, aliquots of cells were exposed to UV (10 mJ/cm^2) and CAT activity was determined 24 h after UV exposure. Upper panel, a representative radioactive image; lower panel, average results of three separate experiments with standard deviation bars.

and cell numbers and viability were determined daily for 6 days. These results demonstrated that cell proliferation and viability were not affected by drug concentrations as high as 2.4 mM. At 4.8 mM of GL, the total cell count after 6 days of cell growth was approximately 50% below that of the control (Fig. 4). The viability of cells was maintained at 95%, indicating that GL was cytostatic but not cytotoxic at this dose. Thus, the IC_{50} of GL for inhibition of cell growth was 4.8 mM.

3.4. Effects of GL pretreatment on UV-induced HIV expression

To examine the effects of GL pretreatment on UV-induced HIV response, the HIV-LTR*cat* cells were grown in medium containing a sub-effective concentration of 0.0012 mM of GL for 7 days, then stimulated for 3 h with fresh medium containing various doses of GL, followed by UV irradiation (10 mJ/cm^2). The cells were harvested for CAT assay at 24 h after the irradiation. A concentration-dependent inhibition was also observed with the GL pretreatment. A significant reduction of CAT activity was observed at 0.024 mM of GL, whereas a more pronounced inhibition was found at 0.06 mM (Fig. 5). The UV-induced CAT activity was completely abolished at a GL concentration of 0.12 mM. No cytotoxicity was observed under this regimen of drug treatment.

3.5. Determination of 50% inhibitory concentration (IC_{50})

The concentration-dependent inhibition of UV-induced CAT activity by GL was observed in cells both with (Fig. 5) and without (Fig. 3) drug pretreatment. The CAT activity was decreased with increasing drug concentrations in both cases but at different rates. Based on the average values of three separate experiments, the IC_{50} values were determined by plotting the total net radioactivity of the acetylated chloramphenicol (after subtracting the background basal level) against the GL concentrations. IC_{50} values with and without pretreatment were 0.04 and 0.38 mM, respectively. Pretreatment with a sub-effective concentration of GL thus resulted in an approximately 10-fold decrease in IC_{50}.

3.6. Kinetics of GL addition on UV-induced HIV expression

Using 1.2 mM concentration of GL, we examined the effects of the addition of GL at varying times after UV

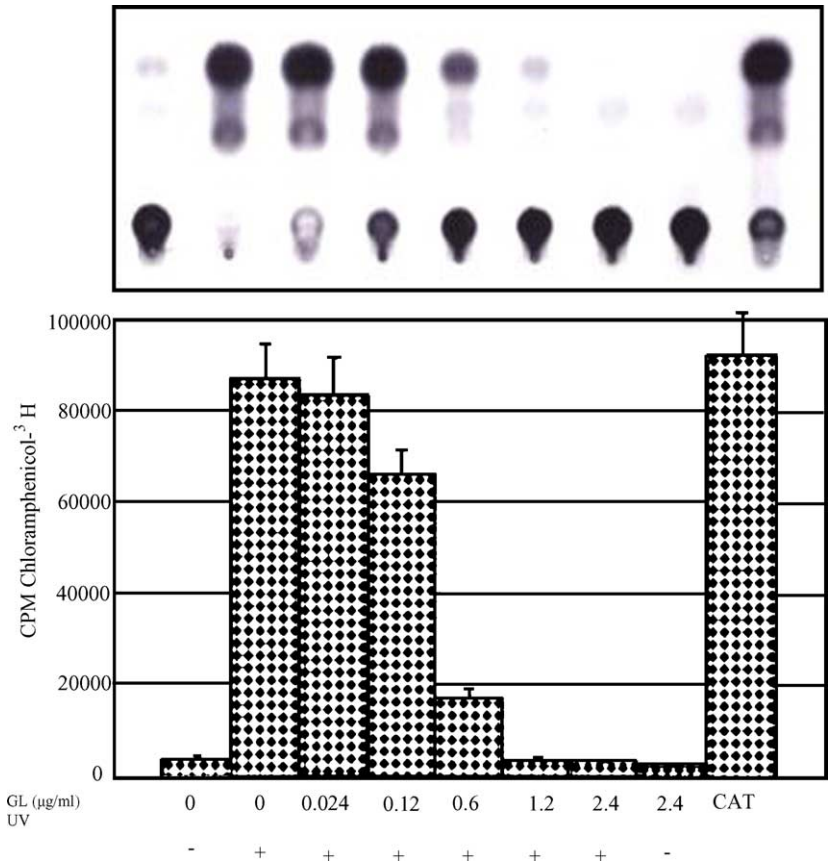

Fig. 3. Concentration-dependent inhibition of UV-induced HIV expression by GL. After stimulation with fresh medium, HeLa HIV-LTR*cat* cells were irradiated with 10 mJ/cm^2 and then immediately treated with various concentrations of GL for 24 h before CAT assay. Upper panel, a representative radioactive image; lower panel, average results of three separate experiments with standard deviation bars.

irradiation. Fig. 6 demonstrates that in the absence of GL, UV induced approximately 30-fold increase in CAT activity. However, the CAT activity was most effectively inhibited by GL if added immediately after UV exposure (0 h). The inhibition was gradually lost as the time of addition was delayed. The inhibition was still evident when GL was added at 5 h after UV exposure, but was completely lost when added at 10 h.

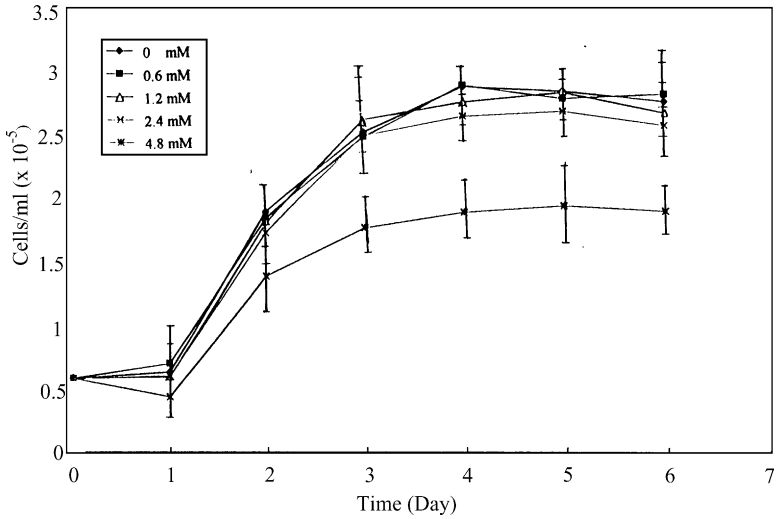

Fig. 4. Effects of GL on cell proliferation and viability. HeLa HIV-LTR*cat* cells were treated with various concentrations of GL, and cell numbers and viability were monitored daily for 6 days. The viability was maintained at 95% for all concentrations tested. The results were from three separate experiments with standard deviation bars.

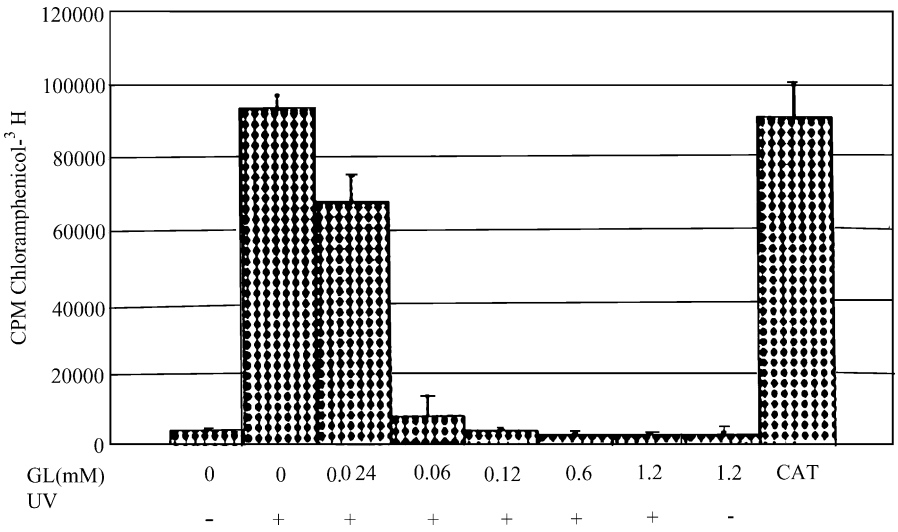

Fig. 5. Effects of GL pretreatment on UV-induced HIV expression. HeLa HIV-LTR*cat* cells were maintained in medium containing a sub-effective concentration (0.0012 mM) of GL for 7 days, stimulated for 3 h with fresh medium containing various concentrations of GL, and followed by UV irradiation (10 mJ/cm^2). The CAT activity was determined at 24 h after UV irradiation. The results were from three separate experiments with standard deviation bars.

3.7. Specificity of GL effects

Histone deacetylase (HAD) inhibitors have been shown to activate the integrated silent viral gene (Chen et al., 1997). To determine the specificity of GL effects on UV-induced HIV expression, the quiescent HeLa-LTR*cat* cells were stimulated with fresh medium with or without GL followed by addition of the HAD inhibitors TSA or SB. The cells were harvested for CAT assay after 24 h of incubation. Both TSA and SB-induced CAT activity to the similar levels as that induced by UV. However, the TSA- and SB-induced CAT activities were not affected by GL at the concentration that completely inhibited UV-induced CAT activity (Fig. 7, panel A). The specificity of the GL effect was also investigated with two potent HIV inducers, TNFα and TPA. Similarly, both TNFα- and TPA-induced CAT activities were not affected by GL (Fig. 7, panel B).

Additional experiments were performed to study the effect of GL on HIV-1 LTR-driven gene expression in H_2O_2-exposed cells and in cells transfected by the cloned *tat* gene construct (pAR*tat*). Exposure of HeLa HIV-LTR*cat* cells to H_2O_2 resulted in activation of the viral LTR, as revealed by intracellular CAT activity. Induction of CAT activity could be observed as soon as 5 h after the oxidative stress, and was maximal after 24 h when the CAT activity was approximately eight-fold above the control level (Fig. 7, panel B). The CAT activity induced by H_2O_2 treatment was abolished by GL, whereas the CAT activity

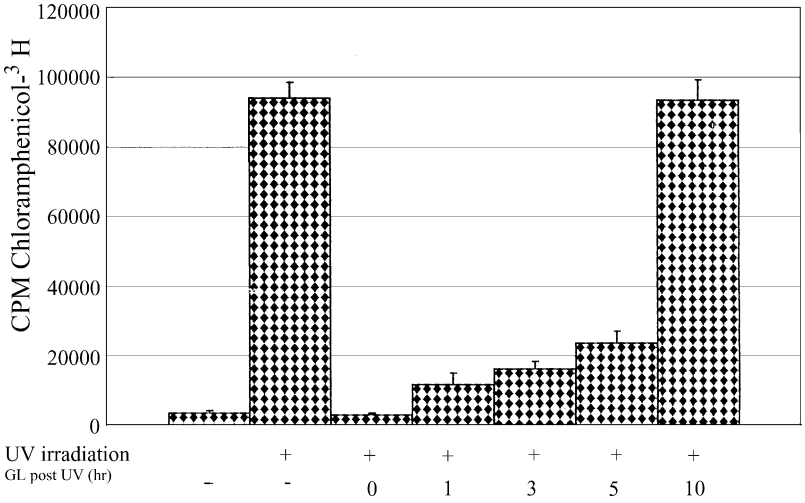

Fig. 6. Kinetics of GL addition on UV-induced HIV expression. The quiescent HeLa HIV-LTR*cat* cells were stimulated with fresh medium for 3 h and were irradiated with UV (10 mJ/cm^2). GL was added to the cells at various times after UV irradiation and cells were harvested 24 h later for CAT assay. The results were from three separate experiments with standard deviation bars.

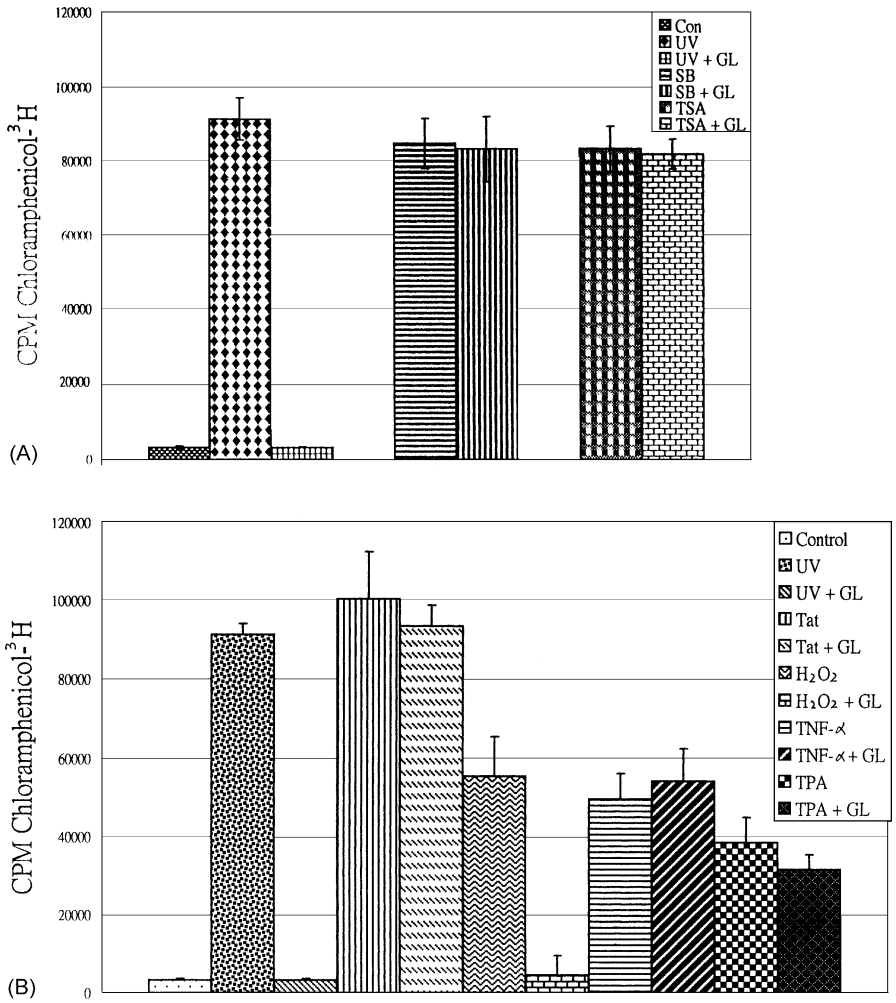

Fig. 7. The specificity of GL effects. The quiescent HeLa HIV-LTR*cat* cells were stimulated for 3 h with fresh medium with (1.2 mM) or without GL, followed by addition of various inducers: TSA (3 μM), SB (50 mM), H_2O_2 (0.5 mM), TNF-α (500 units), and TPA (10 ng/ml). After 24 h of treatment with these drugs, cells were harvested for CAT assays. Exponentially growing cells were transfected with plasmid (pAR*tat*) and cells were harvested for CAT assay after 48 h. The results of SB and TSA are shown in panel A and those of tat, H_2O_2, TNF-α, and TPA in panel B.

of transactivated by tat was not affected by GL treatment (Fig. 7, panel B). These results, taken together, indicate that the first event in HIV-1 reactivation following oxidative stress mediated by UV and H_2O_2 was the LTR transactivation by cellular proteins, which proved to be sensitive to GL.

3.8. Effects of GL on transcription factor profile

Profiling kits were used to identify transcription factor(s) activated by UV stimulation. Fig. 8 shows transcription factor profiling in nuclear extracts from control and UV-activated HeLa HIV-LTR*cat* cells. The control cells exhibited low levels of NF-κB p65, NF-κB p50, c-Fos, CREB-1, ATF2, and c-Rel binding to their target elements. In contrast, cells stimulated with UV produced significant increase in DNA-binding activities of these transcription factors. In the presence of GL, the binding activities of NF-κB p65, NF-κB p50, c-Fos, and c-Rel were completely abolished, but that of CREB-1 and ATF2 were only partially inhibited (Fig. 8, panel A).

The binding specificity between DNAs and the transcription factors was demonstrated by competition assays. Using oligo nucleotides that had the same DNA sequence as the oligo-coated wells as effective competitors for the transcription factor binding, a concentration-dependent competition profile was observed. Representative results are shown in panel B of Fig. 8.

4. Discussion

Previous studies have reported that UV exposure of cells infected with HIV or transfected with HIV reporter genes increases virus-directed gene expression (Valerie et al., 1988). In this study, we further refined and established optimal cell culture conditions for maximal UV induction of HIV-1 expression. We showed that the physiological condition of the

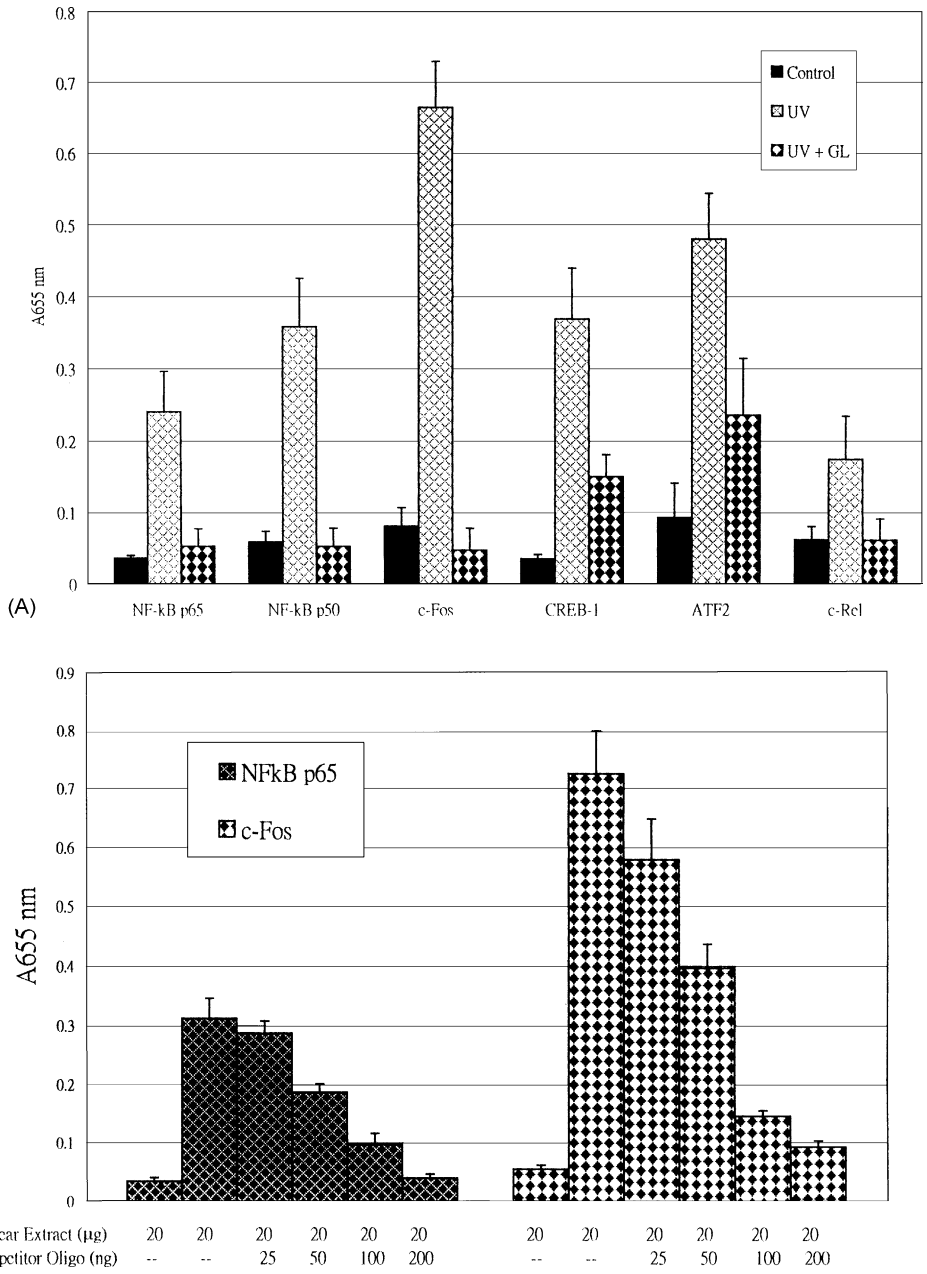

Fig. 8. Transcription factor profiling in nuclear extracts. The TransFactor Profiling Kit-Inflammation 1 (Clontech, Inc.) was used to analyze nuclear extracts from UV-exposed HeLa HIV-LTR*cat* cells in the presence and absence of GL. Panel A shows DNA binding activities of NF-κB p65, NF-κB p50, c-Fos, CREB-1, ATF2, and c-Rel. Panel B shows nuclear extract binding and competition assay data of NF-κB and c-Fos.

cells at the time of UV exposure was one of the critical determinants of the magnitude of response.

We demonstrated that GL, when administered before, immediately after, or up to 5 h after exposure to UV, was able to inhibit UV-induced HIV-LTR-directed CAT activity, and the inhibition was concentrated-dependent. However, if the addition of GL was delayed until 10 h after UV exposure, the inhibition was completely lost. Thus, the first few hours following exposure to UV are critical for the induction of HIV, an event that does not peak until 24 h after the insult. Addition of GL at later times diminished the level of inhibition of the UV-induced HIV response. The inhibitory effect was diminished when GL was washed out of the culture, suggesting that the continuous presence of GL is required for the suppressive response to be effective.

Our results also indicate that GL is a unique inhibitor against UV- and H_2O_2-induced HIV gene expression, since it has no effect on other HIV gene expression inducers. GL is highly selective in its inhibitory effects in that it inhibits UV-induced HIV gene expression at a concentration (0.04 mM), far below the cytostatic concentration (4.8 mM). The marked decrease of IC_{50} observed following pretreatment of the cells at a sub-effective concentration of GL has

potential clinical implications with regard to the eventual effects of GL on the chronic HIV carrier state.

GL has been shown to have many biological effects on mammalian cells, most prominent of which are the anti-viral and anti-inflammatory activities. The anti-inflammatory drug, salicylic acid, has previously been shown to inhibit UV- and *cis*-platinum-induced HIV-1 gene expression (Woloschak et al., 1995). Since salicylic acid inhibits prostaglandin synthesis by inhibiting the cyclooxygenase, it was suggested that the prostaglandins or cyclooxygenase pathways or both are involved in HIV-induction mediated by DNA-damaging agents (Woloschak et al., 1995). The intracellular pathways and the actual inducing signal for the DNA damage response have not yet been identified. It is likely that the inhibition of UV-induced HIV-1 gene expression by GL may act through pathways similar to that of salicylic acid. Ascertaining this possibility will require further study.

The time frame for addition of GL to achieve inhibition of HIV expression is at the onset of the transcription process, when NF-κB and c-Fos are most likely to be activated (Kopp and Ghosh, 1994). Previous work has indicated that NF-κB is activated post-translationally in Hela cells by UV irradiation (Stein et al., 1989; Devary et al., 1993). Valerie et al. (1995b) have demonstrated that transcription factors NF-κB and Ap-1 are activated after UV exposure, which correlate with the increased HIV*cat* steady-state mRNA and CAT activity in HeLa cells stably transfected with HIV-LTR*cat*. To correlate the effects of UV on HIV*cat* transcription with the effects of GL on cellular factors, we analyzed the transcription factors NF-κB p50, NF-κB p65, c-Fos, CREB-1, ATF2, and c-Rel. The results indicated that UV produced a significant increase in the binding of these cellular transcription factors to their target elements. The inhibitory effect of GL on UV-induced CAT activity was associated with a complete inhibition of the binding activities of NF-κB p65, NF-κB p50, c-Fos, and c-Rel but only with partial inhibition of CREB-1 and ATF2.

The assay we described here is based on UV activation of a genetically engineered indicator cell line that contains inducible reporter gene driven by HIV-1-LTR. The advantages of this assay for testing drug effects are: it is specific for HIV, no infectious virus is involved, and it is sensitive and relatively rapid and safe. The establishment of an easy quantitative assay may facilitate the routine screening of compounds that interfere with the HIV-1 gene expression. This assay may prove valuable for the development of new therapeutic approaches, including the ones focusing on specific inhibitors that interact with cellular factors involved in HIV-1 gene expression.

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