Dexamethasone and mifepristone increase retroviral infectivity through different mechanisms

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Solodushko V, Bitko V, Fouty B. Dexamethasone and mifepristone increase retroviral infectivity through different mechanisms. Am J Physiol Lung Cell Mol Physiol 297: L538–L545, 2009. First published June 26, 2009; doi:10.1152/ajplung.00162.2009.—Using adapted retroviruses for gene delivery is a modern and powerful tool in biological research as well as a promising approach for gene therapy. An important limitation for the extensive use of retroviral vectors is the low infection rate in target cells such as pulmonary vascular endothelial cells due to the insufficient infectivity of standard retrovirus supernatants that can only be overcome by complicated methods of virus concentration. This paper describes two easy methods to augment target cell infectivity, first by increasing the retroviral titer in the medium collected from packaging cells by stimulation of viral propagation with dexamethasone, and second, by increasing target cell sensitivity to retroviral infection by the glucocorticoid receptor antagonist, mifepristone. Using this method, we increased the infectivity of pulmonary microvascular endothelial cells from 16% to 85%. We demonstrate that mifepristone increased the susceptibility of target cells to retroviruses without increasing the viral titer of the supernatant. Dexamethasone, but not mifepristone, increased expression of delivered proteins such as GFP that are important for early identification of infected cells. Each, or both step(s), may be included in a standard protocol for retrovirus propagation and infection of target cells.

Materials and Methods

Materials. Dexamethasone (D4902), mifepristone (M8046), hexadimethrine bromide (H9268), and purified monoclonal mouse anti-β-actin antibody [AC-15, horseradish peroxidase (HRP) conjugated] were all purchased from Sigma (St. Louis, MO). Mouse monoclonal anti-green fluorescent protein (GFP) antibody (B-2, sc-9996, HRP conjugated) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). DMEM, EDTA, and L-glutamine were all purchased from Gibco (Grand Island, NY). FBS was purchased from Atlanta Biologicals (Lawrenceville, GA). HyBond-P membrane was purchased from Amersham (Buckinghamshire, England). SuperSignal West Dura was purchased from Pierce (Rockford, IL). Charcoal-stripped FBS (TX73240) was purchased from GeneTex. FuGENE 6 Transfection Reagent (11 814 443 001) was purchased from Roche Diagnostics (Indianapolis, IN).

Cell cultures. Phoenix (ectotropic) packaging cells were purchased from Orbigen (San Diego, CA). Rat pulmonary microvascular endothelial cells (PMVEC) were obtained from our cell culture core. Phoenix and PMVEC were cultured in DMEM, 10% FBS, and 2 mmol L-glutamine and used for experiments at passages 4–9. For some experiments, charcoal-stripped FBS was used in place of normal FBS. All cells were grown in humidified incubators at 37°C in 5% CO2.

Vectors and delivery systems. pBMN-GFP, a retroviral vector that expresses GFP driven by the MMLV’s promoter in Phoenix and other cells, was purchased from Orbigen. Packaging cells were transfected with pBMN-GFP using FuGENE 6 reagent following the manufacturer’s instructions. Stably transfected Phoenix cells were selected by GFP expression using flow cytometry and then used for experiments. For virus propagation, packaging cells were cultured in growth medium for at least 12 h. Virus was collected with cultured medium, filtered, supplemented with hexadimethrine bromide (polybrene) to a final concentration of 4 μg/ml, and applied directly to target cells. The level of viral RNA in collected medium was determined by quantita-
tive PCR using iScript One-Step RT-PCR kit (with SYBR Green, Bio-Rad, 170-8893) using primers for the GFP region of the viral RNA following the manufacturer’s instructions.

Western blotting. Cells were harvested, lysed, resolved in SDS-PAGE, and probed with HRP-conjugated antibodies overnight. Positive antibody reactions were visualized using SuperSignal chemiluminescence detection system (Pierce) according to the manufacturer’s instructions.

Flow cytometry analysis. Cells were harvested by 0.05% trypsin/0.53 mM EDTA digestion, washed, resuspended in cultured medium, and analyzed directly by FACScan in the University of South Alabama Flow Cytometry Core.

Statistical analysis. Data are expressed as means ± SE. Cell growth, infectivity, and changes in fluorescent protein expression were compared using ANOVA combined with Fisher post hoc analysis, with a $P$ value $< 0.05$ considered significant.

RESULTS

Dexamethasone enhances expression of viral proteins and increases retroviral titer. We first tested the ability of dexamethasone to increase retroviral protein production in Phoenix cells with integrated proviruses using GFP as a reporter. We cultured cells for 36 h in DMEM supplemented with 10% FBS and varying concentrations of dexamethasone. We then analyzed the Phoenix cells for GFP expression by flow cytometry. As shown in Fig. 1A, dexamethasone at concentrations of 10 nmol and higher more than doubled GFP levels in these cells. We confirmed this by Western blot analysis of cell lysates obtained from vehicle- and dexamethasone-treated cells (Fig. 1B). To determine if this increase in GFP expression correlated with an increase in viral titer in the supernatant, we performed quantitative RT-PCR on the conditioned medium using primers for the GFP region of the viral RNA. Viral titer increased in a dose-dependent manner reaching a plateau at 10 nmol of dexamethasone (Fig. 1C) suggesting that at this concentration, the proviral LTR (long terminal repeat) promoter is maximally activated by dexamethasone.

While this result demonstrated that dexamethasone could stimulate the retroviral LTR promoter of the provirus, the true measure of retroviral assembly and infectivity is the ability to infect other (target) cells. Therefore, to test whether dexamethasone increased viral production (and infectivity), ecotropic Phoenix cells were grown with increasing concentrations of dexamethasone for 36 h to ~50% confluence. Conditioned medium was then collected and directly applied to rat PMVEC. After 12 h, the conditioned medium was replaced with fresh medium with the same concentration of dexamethasone for an additional 72 h. Cells were then collected and analyzed for GFP expression using flow cytometry. The percentage of GFP-positive (i.e., infected) PMVEC increased as the amount of dexamethasone in the conditioned medium increased. This occurred in a dose-dependent fashion and reached a plateau at 10 nmol where 45% of rat PMVEC was infected (Fig. 2A).

To assess whether dexamethasone had the same stimulatory effect on the LTR promoter of the provirus integrated into the target cells that it did on packaging cells (Fig. 1A), the expression level of GFP in infected cells was also measured. Figure 2B demonstrates that dexamethasone stimulated the LTR promoter of the provirus integrated into the target cells in the same dose-dependent fashion demonstrated in the packaging cells. Dexamethasone had no effect on cell growth over 7 days in either retrovirus-producing or target cells (data not shown).

To determine whether dexamethasone itself increased retroviral activity by facilitating viral infection, we took conditioned medium from Phoenix cells grown without dexamethasone and then added increasing concentrations of dexamethasone. We applied the retroviral-conditioned medium to rat PMVEC for 12 h. The viral supernatants were then replaced with fresh culture medium with the same dexamethasone concentrations for the next 72 h. Cells were then analyzed by flow cytometry to determine the percent of GFP-positive cells. Supplementation of conditioned medium with dexamethasone (in the absence of Phoenix cells) did not increase the percent of infected target cells (Fig. 3A). Consistent with our previous data, however, dexamethasone did increase the intensity of

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Fig. 1. Dexamethasone enhances expression of viral proteins and increases viral release into the supernatant. Phoenix cells were grown in increasing concentrations of dexamethasone for 36 h. A: expression of the retroviral reporter protein GFP as measured by flow cytometry (mean fluorescence per cell, normalized to unstimulated cells). Gray line represents background signal for Phoenix cells without integrated provirus. B: Western blot of cell lysates probed with an anti-GFP antibody in 0 or 100 nmol of dexamethasone-treated Phoenix cells. C: quantitative PCR on the conditioned medium using primers for the GFP region of the viral RNA. N, Phoenix cells without the integrated proviral DNA; $n = 4$ experiments, *$P < 0.05$. 
GFP expression in those cells that were infected (Fig. 3B). This indicates that the addition of dexamethasone at the time of virus propagation, but not after medium collection, increased viral activity. The addition of dexamethasone to collected medium did not alter infection efficiency, but it did boost expression of viral proteins in those target cells that were infected.

Reducing steroid hormones in FBS decreases activation of LTR promoter and reduces viral titer. Since steroid hormones present in serum may activate the LTR promoter at baseline (i.e., before the addition of dexamethasone), we examined whether reducing these steroids in serum would decrease virus propagation and infectivity. We grew Phoenix cells with an integrated provirus expressing GFP to maximum confluence in DMEM supplemented with 10% charcoal-stripped FBS (which has a reduced steroid hormone content) (8) for 72 h. GFP expression in these cells was less than half that of cells exposed to regular FBS (Fig. 4A). Using primers for the GFP region of the viral RNA, we performed quantitative RT-PCR of the conditioned medium and found a decrease in retroviral RNA levels in collected medium obtained from packaging cells grown in charcoal-stripped FBS. The viral titers correlated directly with the GFP levels in Phoenix cells (Fig. 4B). The conditioned medium collected from packaging cells grown in charcoal-stripped FBS demonstrated about a sevenfold decrease in ability to infect target cells compared with medium collected from packaging cells grown in normal serum (Fig. 4C). Charcoal-stripped FBS had no adverse effect on the growth or viability of the packaging cells over 10 days. Also, it was not detrimental to the target cells during a 12-h incubation (data not shown).

The glucocorticoid receptor antagonist, mifepristone, increases target cell infectivity independent of viral titer. The retroviral LTR promoter is known to have hormone response

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Fig. 2. Dexamethasone pretreatment increases both the number of infected target cells and the expression of viral proteins within infected cells. Conditioned medium from Phoenix cells grown in increasing concentrations of dexamethasone was directly applied to rat pulmonary microvascular endothelial cells (PMVEC). A: percentage of GFP-positive cells. B: intensity of GFP expression levels in positive (infected) cells as measured by flow cytometry. Gray line represents background signal for noninfected PMVEC; n = 4 experiments, *P < 0.05.

Fig. 3. Adding dexamethasone to collected viral supernatant does not increase target cell infectivity, but does increase expression of viral proteins in infected cells. Conditioned medium from Phoenix cells grown without dexamethasone was collected and then supplemented with 10, 100, or 1,000 nmol of dexamethasone, before being added to rat PMVEC. A: percentage of GFP-positive cells. B: GFP intensity within positive (infected) cells after 72 h as measured by flow cytometry. Gray line represents background signal for noninfected PMVEC; n = 4 experiments, *P < 0.05.
elements (5, 12), and it appeared that this promoter was stimulated not only by dexamethasone but by steroid hormones within FBS that can be removed by charcoal (Fig. 4). Mifepristone (RU-486) is a glucocorticoid (and progesterone) receptor antagonist that can act as an abortifacient (4). We tested the effect of varying concentrations of mifepristone on retroviral production in Phoenix cells. We grew cells to 50% confluence (as described above) in the presence of increasing concentrations of mifepristone (0–20 μmol), but without dexamethasone. Conditioned medium was then collected and applied directly to PMVEC. After 12 h, the conditioned medium was analyzed using primers for the GFP region of the viral RNA. C: percent of infected target cells (rat PMVEC) incubated with conditioned medium under each condition. Top is a histogram representing the aggregate data from 4 experiments; bottom is a representative example of a single experiment. *P < 0.05.

To determine whether mifepristone could change viral infectivity, the conditioned medium from these Phoenix cells was added to rat PMVEC for 12 h, and the percentage of GFP-positive cells was determined 72 h later. Surprisingly, the presence of mifepristone in the medium increased the susceptibility of target cells to viral infection even though it had no effect on viral titers (Fig. 6A). Mifepristone at 2 μmol and higher concentration more than doubled the percentage of infected cells. Similar to its effect on the packaging cells, mifepristone did not alter GFP expression in infected target cells indicating that unlike dexamethasone, it did not stimulate the proviral LTR (Fig. 6B).

We next tested the effect of adding both dexamethasone and mifepristone to the packaging cell line at the time of viral
propagation. Consistent with our previous results, dexamethasone elevated viral RNA level in the medium from Phoenix cells, whereas mifepristone did not (Fig. 7A). When both dexamethasone and mifepristone were added to the packaging cells at the same time, however, mifepristone completely inhibited the enhancing effect of dexamethasone on virus production (Fig. 7A). This result is likely explained by mifepristone’s ability to compete with dexamethasone for binding sites on the glucocorticoid receptor leading to decreased activation of the LTR promoter (5, 23). Figure 7B demonstrates that mifepristone increased target cell infectivity to a similar degree whether it was added to the packaging cell line at the time of viral propagation or to the conditioned medium after its collection. In contrast, dexamethasone must be added to the packaging cell line to be effective. When both dexamethasone and mifepristone were added to the packaging cell line, the infectivity mirrored that seen with mifepristone alone.

To determine whether either mifepristone or dexamethasone was increasing GFP intensity and expression through direct effects on GFP stability or fluorescence intensity, we repeated these experiments using two other retroviruses, one in which GFP was replaced by DsRed and another in which it was replaced by mCherry. We found a similar effect on target cell infectivity and fluorescence intensity per cell indicating that nonspecific effects of dexamethasone or mifepristone on reporter gene stability were not the explanation for our results (data not shown).

Sequential addition of dexamethasone and mifepristone yields highest target cell infectivity rate. The above data suggested that dexamethasone increased viral titers when

Fig. 5. Mifepristone has no effect on expression of viral proteins or viral titer of supernatant in Phoenix (packaging) cells. Phoenix cells were incubated with increasing concentrations of mifepristone for 36 h. A: expression of the retroviral reporter protein GFP as measured by flow cytometry (mean fluorescence per cell, normalized to vehicle-treated cells). Gray line represents background signal for Phoenix cells without integrated provirus. B: Western blot for GFP of Phoenix cell lysates treated with 0 or 2 µmol mifepristone. C: quantitative PCR on the conditioned medium using primers for the GFP region of the viral RNA. N, conditioned medium from Phoenix cells without the integrated proviral DNA; n = 4 experiments.

Fig. 6. Mifepristone added to the conditioned medium increases the percentage of infected target cells but does not alter the viral protein expression in infected cells. Phoenix cells were grown in the presence of increasing concentrations of mifepristone, and the conditioned medium was then applied to rat PMVEC for 72 h. A: percent of GFP-positive (infected) cells. B: intensity of GFP expression in positive (infected) cells as measured by flow cytometry. Gray line represents background signal for noninfected PMVEC; n = 4 experiments, *P < 0.05.
added to packaging cells, whereas mifepristone increased the infectivity of target cells through another mechanism that was independent of viral titer. This suggested that the highest rate of infectivity should occur when dexamethasone is incubated with packaging cells without mifepristone, followed by the addition of mifepristone to the collected conditioned medium before incubation with target cells. To test this, ecotropic retroviruses expressing GFP were propagated in medium with or without 100 nmol dexamethasone. After collection, the conditioned medium was added to rat PMVEC with or without 2 μmol of mifepristone. After 12 h of infection, the medium was replaced with fresh medium, and target cells were analyzed for GFP after 72 h. Figure 8A demonstrates the percent of infected cells under each of these different conditions. The stepwise addition of dexamethasone and mifepristone increased retroviral infectivity the greatest, from 16% in untreated cells to 85% in sequentially treated cells. Figure 8B is a representation of our flow cytometry data that demonstrates

Fig. 7. Dexamethasone increases target cell infectivity by increasing viral titer, whereas mifepristone increases target cell infectivity independent of viral titer. Phoenix cells were incubated with vehicle (C), 2 μmol mifepristone (M), 100 nmol dexamethasone (D), or both (DM) for 36 h. A: quantitative PCR on the conditioned medium using primers for the GFP region of the viral RNA. B: percent GFP-positive (infected) cells after exposure to the conditioned medium under each condition. Mifepristone and dexamethasone were added either to the packaging cells or to the medium after collection before applying to target cells; n = 4 experiments, *P < 0.05, **P < 0.01. N, conditioned medium from Phoenix cells without the integrated proviral DNA.

Fig. 8. The sequential addition of dexamethasone at the time of virus propagation and mifepristone at the time of infection resulted in the highest rate of target cell infectivity. Conditioned medium from Phoenix cells was applied to target cells (rat PMVEC) for 12 h, and the percent of GFP-positive (infected) cells was determined 72 h later by flow cytometry. A: percent of infected target cells after incubation with conditioned medium in which 2 μmol of mifepristone was added to Phoenix cells (M), 100 nmol dexamethasone was added to Phoenix cells (D), or 100 nmol dexamethasone was first added to the Phoenix cells and then 2 μmol mifepristone was added to the collected medium before application to target cells (DM). C, untreated cells. B: representative flow cytometry tracing depicting percent of infected cells on the y-axis and intensity of GFP expression on the x-axis (data smoothed for graphing). C: conditioned medium was collected and stored in a CO2 incubator at 37°C for the indicated times in the presence of mifepristone, dexamethasone, or both. Each day, this conditioned medium was added to target cells, and the percent of infected cells was determined 72 h later. Infectivity (normalized to fresh conditioned medium with hormones) dropped ~85% per day but was not changed by the presence of dexamethasone or mifepristone; n = 4 experiments, *P < 0.05, **P < 0.01.
that the addition of dexamethasone to the packaging cell line increased both the number of infected target cells (y-axis) and the intensity of GFP within those infected cells (x-axis), whereas the addition of mifepristone increased only the number of infected target cells.

To demonstrate that mifepristone and/or dexamethasone did not increase infectivity by prolonging viral particle survival, we tested the infectivity of conditioned medium with dexamethasone or mifepristone after several days of incubation in a CO2 incubator at 37°C. Figure 8C demonstrates that the number of positive (infected) target cells decreased by ~85–90% over each 24-h time period compared with fresh conditioned medium. This decline was not attenuated by the addition of either dexamethasone or mifepristone.

**DISCUSSION**

The main findings of this paper are: 1) dexamethasone can stimulate the LTR promoter of integrated proviruses in different cell types, 2) dexamethasone increases the viral titer of (Phoenix) packaging cell lines, and this results in an increase in target cell infectivity, 3) mifepristone can increase target cell infectivity independently of retroviral titer, and 4) the sequential addition of dexamethasone and mifepristone is the most effective strategy to increase target cell infectivity in our system.

The family Retroviridae comprises a variety of enveloped RNA viruses, such as endogenous retroviruses, leukemia viruses, and human immunodeficiency virus (HIV)-1. Their replication requires reverse transcription of the viral RNA into linear double-stranded DNA and the subsequent integration of this DNA into the genome of the cell (provirus). To make new virus, the viral DNA genome must first be copied back to RNA using a host enzyme, RNA polymerase II. The LTR region is the control center for gene expression. All of the requisite signals for gene expression are found in the LTR. Retroviral LTRs contain elements that control the level and tissue specificity of transcription and include a hormone response element responsible for stimulating retroviral transcription in the presence of specific steroid hormones. These hormones control many physiological processes by activating specific receptors that act as transcription factors.

In vivo many LTR promoters can be activated by glucocorticoids and progestins through the interaction of the hormone-receptor complex with hormone response elements. Glucocorticoids regulate transcription of provirus through the binding of a glucocorticoid receptor to the glucocorticoid response element that has been described for a number of retroviruses (3, 9, 10, 14, 17, 20, 24), including HIV (15, 18, 22, 25). Although the activation of the proviral promoter by dexamethasone or other steroids has been described (1, 6, 7, 11, 19), the demonstration that this leads to an increase in the formation of active viral particles and ultimately to an increased infectivity of target cells has not. Here we show that dexamethasone cannot only regulate viral promoters, but also increase target cell infectivity by increasing viral titers in the medium of packaging cells.

Although the biological significance of steroid hormones in retroviral cycling is not clear, the advantage of stimulating retroviral production to obtain higher titer virions for gene delivery is obvious. In most protocols for generating retrovirus, the only source of hormones in cell culture is serum. Our experiments using charcoal-stripped medium to remove these steroids indicate how important they are for viral production. We demonstrate that the addition of dexamethasone at just a 10 nmol concentration, however, can markedly increase the viral titer without any obvious adverse effects on cell proliferation or survival. Since the glucocorticoid receptor antagonist, mifepristone, partially blocks the stimulatory effect of dexamethasone on viral production in Phoenix cells, dexamethasone’s effect likely requires binding to the glucocorticoid receptor.

MMLV is widely used as a DNA-delivery vehicle for gene therapy (2). It is a suitable model for studying retroviral function in response to dexamethasone and mifepristone. We were able to increase retroviral infection efficiency by first stimulating the viral promoter in Phoenix cells using dexamethasone and then by adding mifepristone to target cells at the time of virus infection. Each or both steps (using dexamethasone and/or mifepristone) may be included in a standard protocol for retroviral propagation and infection of target cells. Both steps are very easy to perform and did not cause any obvious adverse effects on cell growth or survival in either the packaging or target cells. After the initial infection cycle, dexamethasone and mifepristone may be removed from the medium. Keeping dexamethasone in the medium after infection, however, did increase the expression level of target and reporter proteins. This may be especially useful if target protein(s) are unstable or difficult to transcribe, and it may also facilitate cell analysis and sorting.

We used the pBMN-GFP retroviral system, with ecotropic Phoenix packaging cells for viral propagation, and rat PMVEC as target cells. Since the actual infection efficiency may vary depending on cell type, we also performed infectivity studies on other host (target) cells including human glioblastoma cells (U87MG), rat pulmonary artery endothelial cells, rat pulmonary artery smooth muscle cells, and HeLa cells. We obtained qualitatively similar results. In addition, we demonstrated that dexamethasone and mifepristone had similar effects on ecotropic, amphotropic, and VSV-G based pantropic systems. Because reporter protein levels can be determined by both protein translation and protein stability, we replaced GFP with DsRed or mCherry to show that the type of reporter gene did not alter our results.

We also demonstrate that mifepristone can enhance retroviral infectivity of target cells without increasing retroviral production in packaging cells. This unexpected effect of mifepristone can be used to improve target cell infectivity that is additive to increasing viral titer and can be used to increase the infectivity of already collected and stored (frozen) retroviral stocks. The mechanism through which this viral-independent effect occurs is not clear. Mifepristone may increase the activity of reverse transcriptase or it may facilitate transport of the newly transcribed viral DNA into the nucleus where it is integrated into the host genome.

This observation that mifepristone can increase the infectivity of target cells is not only important in improving target cell infectivity, but may have relevance to retroviral biology. Interest has been generated that mifepristone, acting as an antagonist of the glucocorticoid receptor, might block HIV replication within infected cells. A recent publication demonstrated that in a dose-dependent manner, mifepristone inhibited the viral protein R (Vpr)-mediated transactivation of HIV-1 LTR-
driven gene expression in HeLa cells, raising hope that mifepristone might be a potential adjuvant therapy for HIV infection (23). One publication published over a decade ago, however, demonstrated that mifepristone could stimulate HIV replication depending on cell type (26). Our results suggest that mifepristone might also increase the ability of retroviruses to infect target cells. Strategies using mifepristone to combat retroviral infections clinically, therefore, should be pursued cautiously.

The current studies indicate that using dexamethasone and mifepristone in retroviral systems may significantly increase the infection efficiency of viral supernatants, especially for situations in which baseline infectivity is low. While our results were performed in vitro, such a strategy may be useful to improve infection efficiency in vivo. In addition, the described effects of dexamethasone and mifepristone on retroviral promoter activity and infectivity may be important for understanding the cellular mechanisms regulating retroviral behavior.

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REFERENCES


