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Glucocorticoid Inhibition of the Fibrinolytic Activity of Tumor Cells

Michael Wigler, John P. Ford and I. Bernard Weinstein

Institute of Cancer Research and Departments of Medicine and Microbiology
Columbia University College of Physicians and Surgeons, New York, New York 10032

We have recently discovered that glucocorticoid hormones can rapidly and virtually completely inhibit the production of plasminogen activator by certain cells (in particular, rat hepatoma cells) grown in vitro. Our studies began with the observation that dexamethasone (Dex), a potent synthetic glucocorticoid hormone, induces a phenotypic reversion in mouse L cells from a transformed, or tumor cell-like state to a more tightly regulated growth state (Ford, Wigler and Weinstein, in prep.). (In this paper, "plasminogen activator" refers to a cell factor which, in the presence of serum, leads to the lysis of fibrin in the assay system described by Reich and coworkers [Quigley, Ossowski and Reich 1974; Unkeless et al. 1974].)

METHODS

The cell lines used in the present study have been described in detail elsewhere (Weinstein et al. 1975a,b; Bomford and Weinstein 1972; Yamaguchi and Weinstein 1975; Ford, Wigler and Weinstein, in prep.). Cell cultures were grown as monolayers on plastic petri dishes. Growth media was either Dulbecco's modified minimal essential medium supplemented with 5% fetal calf serum (for 5E, W8 and ts 223 cells) or Ham's F-12 supplemented with 10% fetal calf serum (for HTC, K-16 and H-4 cells). Cells were fed every two days and passaged at low dilution upon reaching confluence.

Assays for plasminogen activator were essentially those described by Reich and coworkers (Unkeless, Gordon and Reich 1974). For measurement of intracellular activity, cell lysates were prepared as follows: Confluent or subconfluent cells in either 5- or 9-cm plastic petri dishes were washed once with phosphate-buffered saline (PBS) and then scraped into 1 ml of 0.2% Triton X-100 in water. Lysates were stored at −20°C until use. Activity was assayed by incubating 0.2 ml of cell lysate, 0.8 ml of 100 mM Tris·HCl pH 8.0 and 0.025 ml of monkey serum (as plasminogen source) at 37°C in ^{125}I-fibrin-

coated 3.5-cm dishes (10 μg/cm^2, 50,000 cpm/dish). At various times thereafter, 0.2-ml aliquots of incubation mixture were sampled and the solubilized ^{125}I measured by counting in a Searle gamma-counter. Control dishes contained 0.8 ml Tris buffer, 0.2 ml 0.2% Triton X-100 in water and 0.025 ml monkey serum. Activity was calculated as the cpm solubilized by the cell lysate $\times$ 100, divided by the total cpm solubilized by trypsin, and expressed as percent. Data were corrected for control dishes in which 2–6% of the cpm was solubilized. The amount of ^{125}I solubilized was approximately a linear function of assay time if no more than 50% of the total fibrin was digested. In any given experiment, cell lysates were made from replicate cultures. Each data point represents the average of two replicate incubations of two replicate cell lysates. The data presented in this paper have not been normalized to the amount of cell protein in lysates, and therefore the results are only semiquantitative.

RESULTS

Figure 1 illustrates the effects of Dex at 10^{-7} M on the appearance of L cells when grown to confluence in plastic petri dishes and on the growth of these cells in suspension in soft agar. Note that untreated monolayer cultures (Fig.

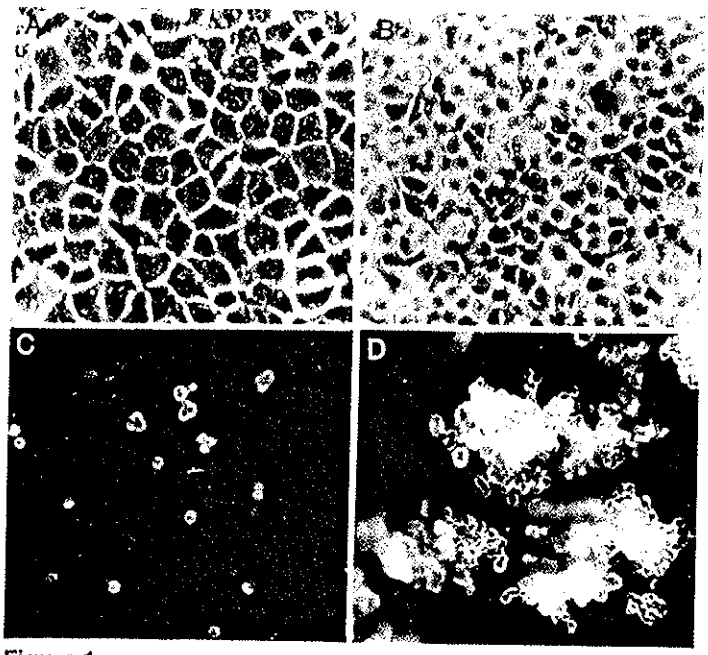

Figure 1
Effects of dexamethasone on L cell morphology and growth. (*A*,*B*) Phase contrast micrographs (90 $\times$) of L cells grown in monolayer to confluence: (*A*) in presence of 2 $\times$ 10^{-7} M dexamethasone; (*B*) control. Darkfield micrographs (36 $\times$) of L cells grown in 0.4% agar suspension: (*C*) in presence of 2 $\times$ 10^{-7} M dexamethasone; (*D*) control.

Table 1
Effects of Dexamethasone on L Cells

1.	Round → flat cells
2.	↓ Piling up
3.	↓ Saturation density
4.	↑ Resistance to trypsinization
5.	↑ Viability at confluence
6.	↓ Growth in soft agar
7.	↓ Production of plasminogen activator

1B) contained cells that were rounded, densely packed and tended to show piling up, whereas treated cells (Fig. 1A) were flat, polygonal and less densely packed. A comparison of C and D in Figure 1 illustrates the marked inhibition of growth in soft agar suspension produced by Dex. Table 1 is a partial list of the effects of Dex that we have observed on the phenotype of L cells (Ford, Wigler and Weinstein, in prep.). We were intrigued that a marked decrease in fibrinolytic activity was found to accompany phenotypic reversion in this system and therefore have pursued this aspect further. Since L cells are not extremely high producers of plasminogen activator, we first screened other lines for a similar effect.

We examined rat hepatoma cell lines because of their well-studied responses to glucocorticoids and the general interest of our laboratory in liver cell lines. Two cell lines (HTC and H-4) derived from hepatomas induced in rats by chemical carcinogens and a rat liver cell line (5E) transformed in culture by murine sarcoma virus (MSV) (Bomford and Weinstein 1972) produced large amounts of plasminogen activator, and this activity was suppressed partially in H-4 and virtually completely in 5E and HTC by 10^{-7} M Dex. We also found that a normal epithelial rat liver cell line (K-16), a tumorigenic cell line (W-8) obtained from K-16, and a temperature-sensitive variant (ts 223) of W-8 (Yamaguchi and Weinstein 1975) all had low plasminogen activator levels even in the absence of Dex.

We chose the HTC cell line for more detailed studies on Dex inhibition of plasminogen activator production for several reasons: HTC has glucocorticoid-inducible tyrosine aminotransferase (TAT), and the response of this system to a variety of steroids has been extensively studied (Baxter and Tomkins 1971). In addition, low concentrations of Dex do not produce a gross inhibition of protein synthesis in this cell line (Thompson, Tomkins and Curran 1966), nor does prolonged culture in the presence of Dex produce growth inhibition (pers. obs.).

Table 2 lists the results when a variety of steroids were assayed for activity in suppressing plasminogen activator production in HTC cells. These results demonstrated that this effect did not occur with all steroids, but appears to be specific for glucocorticoids.

Figure 2 illustrates dose response curves comparing Dex to the less potent compound cortexolone (11 deoxycortisol). The data indicate that Dex was 20 times more potent than cortexolone on a molar basis. The dose response curves are typical for steroid hormones and presumably reflect the satura-

Table 2
Inhibition of Plasminogen Activator by Various Steroids

Addition[a]	*Fibrinolytic activity* (%)	*Inhibition* (%)
None	40	0
Testosterone (10^{-5} M)	38	0
Estradiol (10^{-5} M)	50	0
14-Hydroxy cortexolone (10^{-5} M)	23	43
Cortexolone (10^{-5} M)	3	92
Dexamethasone (10^{-7} M)	1	>97
Cortisol (10^{-6} M)	0	>97
Dibutyryl cAMP (10^{-3} M)	64	0

[a] All test agents added to replicate cultures of HTC cells 24 hours in advance of assaying for intracellular plasminogen activator. See text for details.

tion of high affinity receptors present in the cytoplasm of the HTC cells (Baxter and Tomkins 1971).

Figure 3 indicates the time course of onset of the Dex effect and compares this to untreated cells and cells treated with either actinomycin D (10 μg/ml) or cycloheximide (20 μg/ml). At zero time, media from replicate cultures were replaced with fresh media either containing no additions (control) or the indicated compound. Intracellular plasminogen activator was assayed at the indicated times. The fluctuations in the control are due to the change in the growth medium, which tends to transiently depress activator levels. Cycloheximide produced an almost immediate and rapid fall in activity, indicating

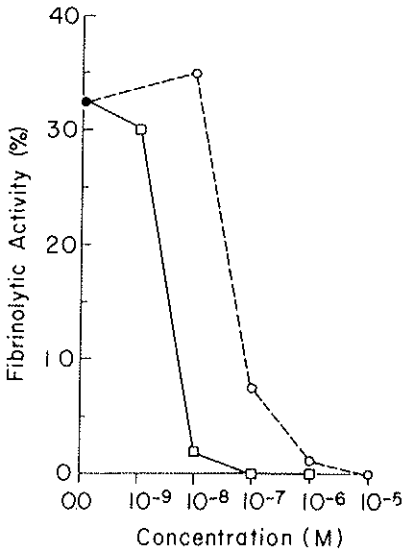

Figure 2
Dose response curves of HTC cells to dexamethasone and cortexolone. HTC cells were grown as replicate cultures, and subconfluent cultures were treated for 24 hours with either dexamethasone (□) or cortexolone (○) at the indicated concentrations before assaying for intracellular plasminogen activator. (●) Indicates activity of untreated cultures.

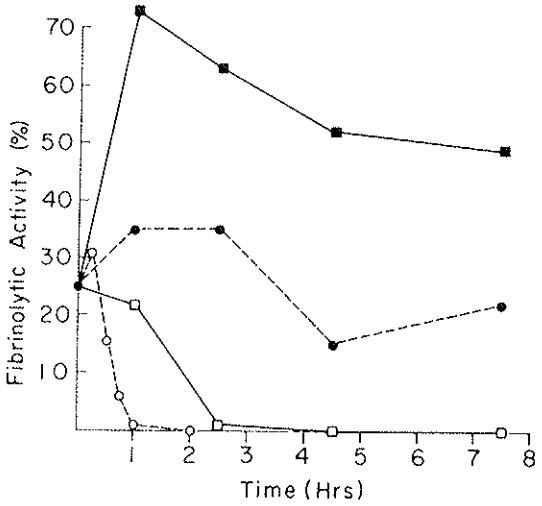

Figure 3

Time course of dexamethasone effect compared to that of various inhibitors. At zero time, HTC cells in replicate cultures were refed fresh growth medium with either 10 μg/ml actinomycin D (■), 20 μg/ml cycloheximide (○), 10^{-7} M dexamethasone (□), or fresh medium without additions (●). At the times indicated on the abscissa, cultures were assayed for intracellular plasminogen activator.

a short intracellular half-life of preformed plasminogen activator. The response to Dex, however, revealed a lag of about one hour followed by a very rapid decline in activity, so that by 2.5 hours, the level was almost undetectable. As can be seen, actinomycin D produced a 2–3-fold elevation in plasminogen activator. Based on these results, we have tentatively concluded that Dex exerts its inhibitory effect on plasminogen activator production at the level of translation (or possibly posttranslation), rather than at the level of transcription.

Data presented in Table 3 indicate that the Dex effect was dependent on de novo transcription. Replicate cultures were either untreated at 0 hours or treated with the indicated agents at 0 or 1 hour, and the intracellular levels of plasminogen activator were measured at 5 hours. Actinomycin D (10 μg/ml), as noted above, and cordycepin (40 μg/ml) led to a 2–5-fold elevation in plasminogen activator. The latter concentration of cordycepin is known to block processing of poly(A)-containing mRNA (Darnell et al. 1971). When either actinomycin D or cordycepin was given one hour prior to Dex, the ability of Dex to decrease the intracellular level of plasminogen activator was completely blocked.

Dex inhibition of plasminogen activator was reversible, as can be seen in Figure 4. Cells were either untreated or pretreated for 4, 24 or 72 hours with Dex. At zero time, media were removed and replaced with fresh media with-

Table 3
Effects of Drug Combinations

Addition		
0 hr	*1 hr*	*% Activity*
None	none	8
None	Dex	1
Act	none	18
Act	Dex	17
Cord	none	44
Cord	Dex	47

At the indicated times, replicate cultures of HTC cells received 10^{-7} M dexamethasone (Dex), 10 μg/ml actinomycin D (Act) or 40 μg/ml cordycepin (Cord). At 5 hours, cells were assayed for intracellular plasminogen activator.

out Dex. At 4, 12, 24 and 48 hours later, the intracellular levels of plasminogen activator were measured. It is apparent that following the removal of Dex, there was a gradual return of plasminogen activator. A curious feature is that recovery was slower in those cells pretreated with Dex for longer periods of time. The underlying mechanism for this is not known at present, but the kinetics of the return of activity are not entirely compatible with the accumulation of a single inhibitor.

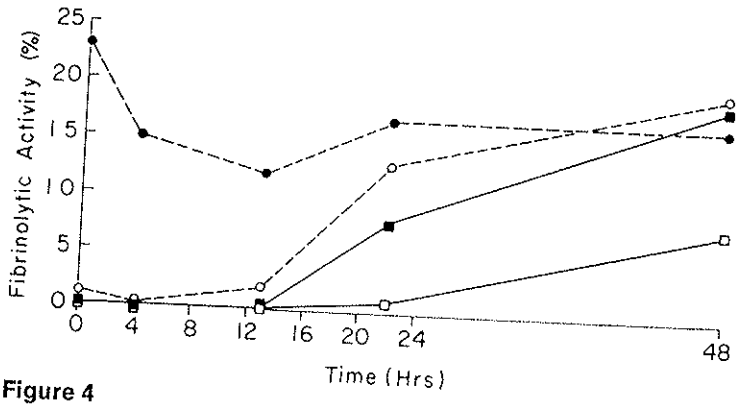

Figure 4
Recovery of activity after removal of dexamethasone. HTC cells in replicate cultures were either pretreated with 2×10^{-7} M dexamethasone for 72 (□), 24 (■) or 4 hours (○), or were untreated (●). At zero time, all cultures were washed three times with PBS and refed growth medium without dexamethasone. Cultures were refed after 24 hours. At the indicated times, cultures were assayed for intracellular plasminogen activator.

DISCUSSION

The following is our currently favored model for explaining Dex inhibition of plasminogen activator production. Dex enters the cell, combines with the cytoplasmic glucocorticoid receptor, and then passes into the nucleus where the receptor-hormone complex causes increased production of an RNA species that directly blocks (or codes for a protein that blocks) translation of the plasminogen activator mRNA. This proposal is based on the following interpretation of the effects of actinomycin D and cordycepin; namely, that the mRNA for the plasminogen activator is stable for at least 7–8 hours, a span in excess of the time needed for occurrence of the Dex effect. The possibility that actinomycin D and cordycepin both artifactually increase the longevity of plasminogen activator mRNA has not been ruled out.

The model proposed is similar to that which Tomkins and others used to explain the observation that actinomycin D blocked the deinduction of TAT synthesis in cells preinduced with Dex (Tomkins et al. 1972). Other models compatible with the present data have not been excluded. Further studies are required to elucidate possible direct or indirect effects of Dex on plasminogen activator translation, posttranslational modification or degradation, or the production of specific inhibitors. We must emphasize that the addition of Dex to the assay system itself does not inhibit fibrinolysis, and therefore, the Dex effect is exerted at the cellular level.

Our results raise the question of whether the mechanisms that maintain the lower level of plasminogen activator in certain normal cells, when compared to their transformed counterpart (Unkeless et al. 1974), are similar to those that mediate the glucocorticoid-induced decrease in plasminogen activator we have observed in transformed liver cells. The effects of actinomycin D on plasminogen activator levels in chick fibroblasts transformed by a temperature-sensitive strain of Rous sarcoma virus (observed by Rifkin, Beal and Reich, this volume) suggest that there are similarities.

Acknowledgments

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