# Dexamethasone Stimulation of Metallothionein Synthesis in HeLa Cell Cultures

Abstract. *HeLa cells in culture synthesize metallothionein. To investigate the effects of glucocorticoids on metallothionein synthesis we adapted HeLa cells to growth in a defined medium lacking hydrocortisone. In this defined medium, containing 1.5 × $10^{-6}$M $Zn^{2+}$, dexamethasone ($10^{-6}$M) caused a five- to sixfold increase in the synthesis of metallothionein. Progesterone ($10^{-5}$M), a known antagonist of glucocorticoids, inhibited this response by 50 percent.*

Metallothioneins (MT's) are low-molecular-weight proteins that are unusual in their high cysteine content (25 to 30 percent) and their high affinity for $Zn^{2+}$, $Cd^{2+}$, $Hg^{2+}$, $Ag^{+}$, and $Cu^{+}$ (*1*). Since they were first demonstrated in equine kidney, MT's have been identified in a variety of different species (*2*). These compounds are induced primarily in the liver and kidney of animals in response to $Cd^{2+}$, $Zn^{2+}$, $Hg^{2+}$, $Ag^{+}$, or $Cu^{+}$ administration (*3*). Recently, Oh *et al.* found that a variety of stresses also stimulate MT synthesis (*4*). Stress is known to increase the concentrations of circulating adrenal glucocorticoid hormones (*5*).

Cox and co-workers (*6*, *7*) found that glucocorticoids increase the uptake of $Zn^{2+}$ by HeLa cell cultures. The effect was specific to $Zn^{2+}$ and was inhibited only by sulfydryl reagents and $Cd^{2+}$. The stimulation of $Zn^{2+}$ uptake by glucocorticoids is dependent on RNA and protein synthesis (*8*). By using HeLa cells grown in a serum-free chemically defined medium containing hydrocortisone (HC) (*9*) and a rat liver cell line grown in serum-free medium, our laboratory demonstrated the induction of MT by both $Cd^{2+}$ and $Zn^{2+}$ (*10*, *11*). The proteins induced in the clonal cell culture lines were characterized as MT's by (i) their inducibility by both $Zn^{2+}$ and $Cd^{2+}$, (ii) binding of $^{109}Cd^{2+}$ and $^{65}Zn^{2+}$, (iii) labeling with [$^{35}$S]cysteine, (iv) elution profiles on Sephadex G-75, and (v) cochromatography with authentic purified MT on ion exchange columns (*10*, *11*).

One explanation for the induction of MT by stress in rats (*4*) and for stimulation of $Zn^{2+}$ uptake in HeLa cell culture by glucocorticoids (*6–8*) may be that the increase in MT synthesis is mediated by glucocorticoids. We now present evidence that dexamethasone, a synthetic glucocorticoid, stimulates MT synthesis in HeLa cell cultures and that progesterone, an antagonist of glucocorticoids (*7*), inhibits this response.

Hydrocortisone is normally present at a concentration of $10^{-7}M$ in the defined medium used for HeLa cells (*9*). To measure MT synthesis in the complete absence of glucocorticoids we adapted the cells to growth in HC-free medium. The trace metal solution used by Hutchings and Sato (*9*) was also left out of our medium. HeLa cells previously grown in HC-containing medium (*10*) were transferred to HC-free medium and subcultured at least three times (at a 1:10 dilution) in HC-free medium before use. The cells used in the experiments reported here were thus in HC-free medium for a minimum of ten generations. We now maintain HeLa cells routinely in HC-free medium. Isotopic labeling, harvesting, processing, and gel filtration of the cell extracts were as reported previously (*10*, *11*), except that 50 m$M$ $(NH_4)_2CO_3$ buffer, $p$H 8.5, was used instead of the tris-HCl buffer. Protein in cell extracts was assayed by dye binding (*12*).

Although HC was included in the original defined medium for HeLa cells (*9*), it is apparently necessary only for optimal cell growth; there is no absolute requirement for HC. HeLa cells grown in HC-free medium exhibited exponential growth, with only a slight increase in doubling time from 38 hours (in the presence of HC) to 46 hours. The concentration of $Zn^{2+}$ in this HC-free medium was $1.5 \times 10^{-6}M$ (*10*).

The gel filtration elution profiles in Fig. 1, A and B, show three peaks of radioactivity. The first peak consists of high-molecular-weight proteins eluting in the void volume and representing the

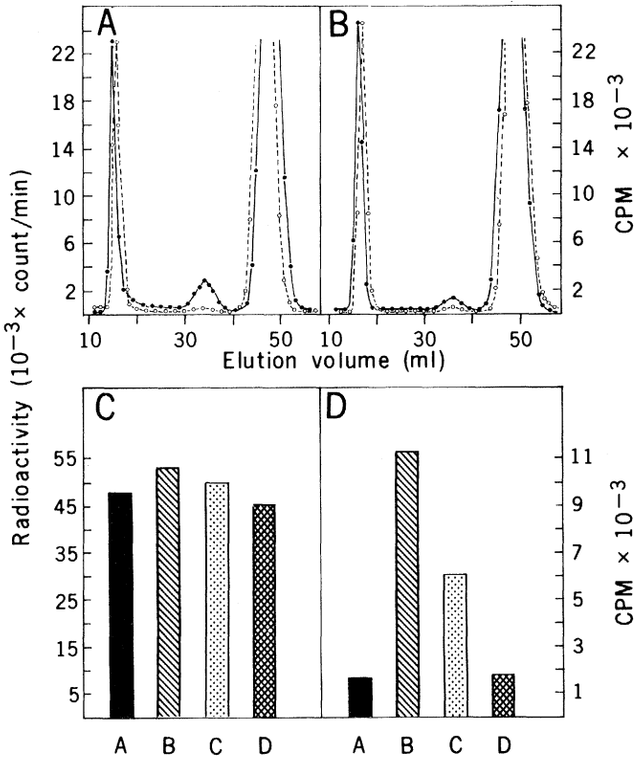


Fig. 1. Effects of dexamethasone and progesterone on MT synthesis in HeLa cells. Confluent cultures of HeLa cells, grown on 100-mm culture plates in 10 ml of HC-free defined medium, were incubated for 24 hours with the different hormones and 10 $\mu$Ci of [$^3$H]cysteine. Hormones were added in 10 $\mu$l of 95 percent ethanol; the control plate also received 10 $\mu$l of ethanol. Cells were harvested and the soluble proteins were chromatographed on Sephadex G-75 columns (1.1 by 50 cm). (A) Sephadex G-75 elution profile of control cells (○) and cells incubated with $10^{-6}M$ dexamethasone (●). (B) Sephadex G-75 elution profile of cells incubated with $10^{-5}M$ progesterone (○) and cells incubated with $10^{-6}M$ dexamethasone and $10^{-5}M$ progesterone (●). (C) Effect of steroid hormones on synthesis of high-molecular-weight soluble proteins. The areas under the peak eluting initially from the Sephadex G-75 column are integrated. Bars: *A*, control; *B*, $10^{-6}M$ dexamethasone; *C*, $10^{-6}M$ dexamethasone and $10^{-5}M$ progesterone; and *D*, $10^{-5}M$ progesterone. (D) Effects of steroid hormones on MT synthesis in HeLa cells. The areas under the second peak to elute from the Sephadex G-75 column are integrated. Bars: *A*, control; *B*, $10^{-6}M$ dexamethasone; *C*, $10^{-6}M$ dexamethasone plus $10^{-5}M$ progesterone; and *D*, $10^{-5}M$ progesterone. All data are expressed as counts per minute per milligram of protein applied to the column.


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majority of soluble HeLa cell proteins labeled by [$^3$H]cysteine. We have previously characterized the second radioactive peak, with an elution volume to void volume ratio of 2, as MT (*10*). The third peak is composed of low-molecular-weight cysteine compounds and free cysteine eluting in the included volume of the gel.

Incubation of HeLa cells for 24 hours with $10^{-6}M$ dexamethasone caused a marked increase in incorporation of [$^3$H]cysteine into the MT peak, when compared to controls with no glucocorticoid (Fig. 1A). Dexamethasone ($10^{-6}M$) caused a sixfold increase in MT synthesis over the control (Fig. 1D). Incubation of HeLa cells with $10^{-5}M$ progesterone had no effect on the incorporation of [$^3$H]cysteine into MT (Fig. 1B). Inclusion of $10^{-5}M$ progesterone in the incubation mixture together with $10^{-6}M$ dexamethasone reduced the incorporation of [$^3$H]cysteine into MT (Fig. 1B) when compared to the large increase seen with $10^{-6}M$ dexamethasone alone (Fig. 1A). Inhibition of the response to $10^{-6}M$ dexamethasone by $10^{-5}M$ progesterone was close to 50 percent (Fig. 1D). This inhibition by progesterone suggests that dexamethasone exerts its effect by binding to the glucocorticoid receptor, since progesterone is known to compete with binding of glucocorticoids to their receptor (*13*). Cox and Ruckenstein (*7*) reported complete inhibition by $10^{-5}M$ progesterone of the stimulation of $Zn^{2+}$ uptake achieved by $10^{-6}M$ HC. The partial inhibition observed in our experiment may be due to the stronger potency of dexamethasone as an inducer, since it is not metabolized. Both progesterone and HC are metabolized. Neither progesterone nor dexamethasone treatment had any marked effect on the incorporation of [$^3$H]cysteine into high-molecular-weight soluble proteins of HeLa cells (Fig. 1C).

The increased synthesis of MT in HeLa cells in response to incubation with dexamethasone might be explained as follows. Dexamethasone may increase the uptake of $Zn^{2+}$ in HeLa cells. This might then increase the availability of $Zn^{2+}$ to the metal-mediated MT induction mechanism, when the cells are grown in concentrations of metal less than optimal for maximal MT induction. The uptake of $Zn^{2+}$ in primary cultures of parenchymal liver cells, the main organ for MT synthesis, can be stimulated by dexamethasone (*14*). Alternatively, the stimulation by dexamethasone of [$^3$H]-cysteine incorporation into MT may be a direct effect of dexamethasone on MT metabolism. Dexamethasone might directly either stimulate MT synthesis or inhibit its degradation.

Ten years ago Cox (*6*) proposed, as a possible mechanism for $Zn^{2+}$ uptake, the existence of a $Zn^{2+}$ carrier or $Zn^{2+}$-binding molecule containing sulfhydryl groups. He proposed that prednisolone (another glucocorticoid agonist) "may enhance $Zn^{2+}$ uptake in certain mammalian cell cultures by increasing the level or activity of carrier molecules." Our studies suggest that MT is this $Zn^{2+}$-binding molecule.

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### References and Notes

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## "Spontaneous" Neoplastic Transformation in vitro: A Form of Foreign Body (Smooth Surface) Tumorigenesis

Abstract. *Explants of subcutaneous connective tissue from adult BALB/c mice into plastic petri dishes were serially subcultured and tested for tumorigenicity in two ways: by the subcutaneous implantation of cells attached to plastic plates (1 by 5 by 10 millimeters), and by the subcutaneous injection of cells suspended in saline. Cells grown in vitro for 18 or more days before being implanted attached to a plastic plate ($2.4 \times 10^4$ to $3.4 \times 10^5$ cells per plate) formed tumors after 24 to 79 weeks. The latent period before tumor appearance correlated inversely with the time spent by the cells in tissue culture. Cells inoculated in saline suspension (10 to 100 times the above number per plate) did not form tumors until after 84 days in vitro; plates alone did not induce tumor formation within more than 1 1/2 years of implantation. The tumors arising from the plate-attached cells were transplantable without plates and histologically appeared to be undifferentiated sarcomas. It is well established that smooth-surfaced foreign bodies, regardless of their chemical composition, will produce sarcomas when transplanted subcutaneously in rodents. We interpret our data, particularly the decrease in tumor latent period with time spent in tissue culture, as indicating that a smooth surface was acting as a carcinogen first in vitro (the surface of the tissue culture dish) and then in vivo (the surface of the plastic plate).*

The etiology of "spontaneous" neoplastic transformation in tissue culture exhibited by mammalian cells from a number of species, particularly rodents, has not been determined (*1*). Neoplastic transformation occurs in cells grown in serum-free medium (*2*), and in the absence of oncornavirus gene products (*3*). Stimulated particularly by the work of Brand on plastic film tumorigenesis in mice (*4*) we tested the hypothesis that "spontaneous" neoplastic transformation of mouse cells in vitro is a form of foreign body (smooth surface) tumorigenesis. Plastic film is representative of a large number of chemically different substances that are sarcomagenic only if they possess an extended flat surface. For example, subcutaneous implants of glass, metals, plastics, and silicone rubber all produce sarcomas in sheet form but not in particulate form (*5*). We postulated that just as association in vivo between the smooth surface of a plastic film implant and subcutaneous connective tissue could give rise to tumors,


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