Hormonal Regulation of Zinc Metabolism in a Human Prostatic Carcinoma Cell Line (PC-3)

Penelope J. Giles and Robert J. Cousins

Department of Nutrition, Rutgers University, The State University of New Jersey, New Brunswick, New Jersey 08903

ABSTRACT

Cells from a human prostatic adenocarcinoma cell line (PC-3) were maintained in M199 medium, with and without 5% Bobby calf serum, to examine aspects of cellular zinc metabolism. Cultures contained 0.5 to 1.0 x 10^6 cells/dish. Enhanced 65Zn accumulation in response to dexamethasone was observed after 24 hr in culture. The rate of efflux of accumulated 65Zn was not influenced by this hormone. Dihydrotestosterone had no effect on the accumulation of 65Zn by whole cells. Between 60 and 75% of the accumulated 65Zn was found in the cytosol (168,000 x g supernatant) fraction. Analysis of zinc distribution in cytosol by gel filtration chromatography revealed two major zinc-containing peaks, one comprised of high-molecular-weight zinc-containing proteins and a low-molecular-weight fraction that eluted identically to purified metallothionein (MT). Ion exchange chromatography of this low-molecular-weight fraction resolved it into two components comparable to the major forms of MT purified from liver. Prior to differential 65Zn accumulation (12 hr), increased 65Zn was bound to the MT-like fraction in dexamethasone-treated cells and increased [35S]cystine was incorporated into this protein fraction. Other steroid hormones (dihydrotestosterone, hydrocortisone), as well as prostaglandin E2, resulted in an increased amount of bound 65Zn in the MT fraction, while estradiol and cortisone had no effect. Incubation of cells with actinomycin D or cycloheximide blocked the stimulatory effect of dexamethasone on the prostatic MT-like fraction. These experiments suggest that the MT genes are expressed in prostatic carcinoma cells. Moreover, they show that various aspects of prostatic zinc metabolism, particularly those related to MT, are influenced by certain steroid hormones, namely dexamethasone and dihydrotestosterone.

INTRODUCTION

The concentration of zinc is extremely high in the prostate gland (24, 32) and its secretions (23). This high concentration is not accounted for by the zinc content of metalloenzymes alone, since these contain only a small percentage of the total prostate zinc (8). In spite of this high zinc content, relatively little is known about the mechanisms by which prostatic cells maintain homeostatic control of zinc (2). Animal experiments have shown that prostatic zinc is under hormonal control (11, 18, 26, 28). Recent findings indicate that, in prostatic cells, zinc may influence androgen metabolism (10, 33) and androgen receptor action (14, 16, 22, 29). One recognized biochemical feature of carcinoma of the prostate is the low zinc concentration in malignant tissue compared with the hypertrophied or normal gland (12). Habib et al. (15) have recently shown a strong correlation between zinc and the ratio of DHT to testosterone in prostatic tissues of different pathological status. These results indicate that androgen and zinc relationships might be used as an index for the proliferation of the prostate gland.

Most information available on the nature of prostatic neoplasia has been obtained from clinical observations and studies on animal models. However, few good animal models exist for prostatic carcinoma due to its limited occurrence, specifically in humans and dogs (30). The value of cell culture models for human prostatic tissue has been recognized for many years (25), but development of such models has been impeded by technical difficulties including cross-contamination of putative prostatic lines with HeLa cells (27). Recently, isolation of 2 prostatic adenocarcinoma lines was reported, and these appear to be the first authentic established prostatic lines available (20, 31). This paper describes the results of experiments on the metabolism of zinc in the PC-3 cell line.

MATERIALS AND METHODS

Materials. M199 medium, Hanks' balanced salts solution, and Bobby calf serum were obtained from Grand Island Biological Co. (Grand Island, N. Y.). Chromatography media were from Pharmacia Fine Chemicals, Inc. (Piscataway, N. J.). All other biochemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Radioisotopes. Carrier-free 65Zn was obtained from Amersham Corp. (Arlington Heights, Ill.). L-[35S]cystine (446 Ci/mmol) was purchased from New England Nuclear (Boston, Mass.).

Cell Culture. PC-3 cells were obtained from American Type Culture Collection (Rockville, Md.). The cells were maintained in 75- or 150-sq cm polystyrene tissue culture flasks in M199 supplemented with 5 to 10% Bobby calf serum, 20 μg streptomycin sulfate per ml, 100 units penicillin G per ml, and sodium bicarbonate to pH 7.2. Zinc content of the media varied from 0.45 to 1.2 μg/ml.

Zinc Accumulation. PC-3 cells were resuspended with 0.25% trypsin, and 0.5 to 1.0 x 10^6 cells were seeded in 60-mm tissue culture dishes and incubated at 37° with 5% CO2. To initiate the experiment, spent media and detached cells were removed by aspiration and replaced with fresh media containing 65Zn (0.5 to 2.0 μCi/dish) and agents being tested for their effect on zinc accumulation. After specific periods of time, radioactive medium was removed from each dish by aspiration, and the cell surface was washed twice with 4 ml of ice-cold 10 mM HEPES-buffered 0.9% NaCl (pH 7.4) containing 10 mM EDTA to remove residual medium and nonspecifically bound zinc (5). Initial
studies showed that 2 washes effectively reduced the quantity of metal nonspecifically associated with the cell surface. All the added radioactive activity was recovered in the media, the washes, and the cell lysate using this procedure.

The quantity of $^{65}$Zn accumulated by cells was measured by liquid scintillation counting. The cells were lysed by addition of 1.5 ml of 0.5% deoxycholate (in 0.4% NaCl) to each dish and scraped from the surface with a rubber policeman. The suspension was added to 15 ml Scintiverse (Fisher Scientific Co., Pittsburgh, Pa.), and the quantity of $^{65}$Zn was measured by liquid scintillation counting at an efficiency of 30%. Viable cell number was determined from control plates by counting, on a hemocytometer, those cells which excluded trypan blue.

Distribution of Accumulated Zinc and Amino Acids in Cell Cytosol. Cells were incubated for specific periods of time in medium containing $^{65}$Zn (0.5 to 2.0 $\mu$Ci/dish) and the agent being tested for its effect on zinc distribution. Steroid hormones and PGE$_2$ were added at 10$^{-6}$ M; inhibitors CX and actinomycin D were added at 10 and 1 $\mu$g/ml final concentration, respectively. The radioactive medium was removed, and the cell surface was washed twice with 4 ml ice-cold 20% HEPES-buffered Hanks’ salts solution. Cells were scraped from the surface of 5 replicate dishes with a rubber policeman. Next the cells were thawed and disrupted by 15 up-down strokes with a motor-driven glass-Teflon Potter-Elvejhem homogenizer. Cellular particulate matter was removed by centrifugation at 166,000 $\times$ g for 60 min. The cytosol preparations (supernatant) were removed and stored in liquid nitrogen until analyzed. Protein concentrations of the cytosol preparations were determined by the micro Bio-Rad procedure (1), and 1 ml of the cytosol preparations was separately applied to a 1.6- x 30-cm column packed with Sephadex G-75 equilibrated previously with 10 mM Tris-acetate buffer, pH 8.6. One-ml fractions were collected at a rate of 20 ml/hr, and the $^{65}$Zn content of each fraction was determined by liquid scintillation counting.

Similarly, in other experiments, cells were incubated in medium containing $^{65}$Zn (0.5 to 2.0 $\mu$Ci/dish) and/or [35S]cystine (2 to 4 $\mu$Ci/ml) final concentration, respectively. The radioactive medium was removed, and the cell surface was washed twice with 4 ml ice-cold 20% HEPES-buffered Hanks’ salts solution. Cells were scraped from the surface of 5 replicate dishes with a rubber policeman. Next the cells were thawed and disrupted by 15 up-down strokes with a motor-driven glass-Teflon Potter-Elvejhem homogenizer. Cellular particulate matter was removed by centrifugation at 166,000 $\times$ g for 60 min. The cytosol preparations (supernatant) were removed and stored in liquid nitrogen until analyzed. Protein concentrations of the cytosol preparations were determined by the micro Bio-Rad procedure (1), and 1 ml of the cytosol preparations was separately applied to a 1.6- x 30-cm column packed with Sephadex G-75 equilibrated previously with 10 mM Tris-acetate buffer, pH 8.6. One-ml fractions were collected at a rate of 20 ml/hr, and the $^{65}$Zn content of each fraction was determined by liquid scintillation counting.

Ion Exchange Chromatography. DEAE-Sephadex A-25 was equilibrated with 20 mM Tris-acetate, pH 7.4, and packed into a 1.5- x 30-cm column (7). Fractions that eluted from the gel filtration columns in the same region as standard rat liver MT were pooled and applied to the DEAE-Sephadex column. After washing with 40 ml of starting buffer, the sample was eluted with a linear gradient (total volume, 240 ml) of 20 to 200 mM Tris-acetate, pH 7.4, at a rate of 20 ml/hr. Three-ml fractions were collected and assayed for $^{65}$Zn.

RESULTS

Zinc Accumulation by Whole Cells. The quantity of zinc accumulated by whole PC-3 cells was significantly increased by the addition of $10^{-6}$ M DX, a synthetic glucocorticosteroid hormone. Although there was a slight increase consistently observed after 18 hr of culture, the difference was not statistically significant ($p > 0.05$) until 24 hr (Chart 1). Addition of insulin (1 $\mu$g/ml) alone had no effect on zinc accumulation at 18 hr (Table 1). Addition of $10^{-6}$ M DHT did not influence the relative zinc accumulation after 18 hr (Table 1) or after 24 hr in culture (data not shown). In terms of absolute amount of zinc, control cells had accumulated 70 pmol Zn per 10$^{6}$ cells at 18 hr of culture. This represents about 50,000 cpm of $^{65}$Zn accumulated.

Incubation of cells in the presence of CX decreased total accumulation of $^{65}$Zn in whole cells by 50% after 18 hr of incubation (Table 1). [3H]Serine, [3H]lysine, and [3H]glucose incorporation into trichloroacetic acid precipitable proteins was decreased by 80% after only 2 hr of incubation with the drug (data not shown).

A portion of the $^{65}$Zn accumulated by whole cells during 18 hr of culture was readily exchangeable with medium components as shown in Chart 2. Approximately 50% of the $^{65}$Zn was lost after 5 hr. Although the decreases in the cellular level of previously accumulated $^{65}$Zn occurred at a similar rate in both control and DX-treated cells, the latter retained a greater quantity of zinc throughout the exchange period. In both control and DX-treated cultures, the rate of exchange was highest during the first 2 hr.

When the cells were disrupted by homogenization and the cytoplasmic fraction was prepared by centrifugation, 60 to 70% of total accumulated zinc was present in the soluble cytoplasmic (cytosol) fraction. The amount of zinc accumulated per mg cytosol protein was 1.3 ± 0.1 (S.D.) times more in DX-treated cells and 1.3 ± 0.2 times more in DHT-treated cells than in the cytosol fraction of control cultures.

## Table 1

<table>
<thead>
<tr>
<th>Substance added</th>
<th>% of control $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100 ± 6.6</td>
</tr>
<tr>
<td>Insulin</td>
<td>95 ± 0.3</td>
</tr>
<tr>
<td>DX</td>
<td>108 ± 10.2</td>
</tr>
<tr>
<td>DX + insulin</td>
<td>112 ± 3.3</td>
</tr>
<tr>
<td>DHT</td>
<td>90 ± 10.5</td>
</tr>
<tr>
<td>CX</td>
<td>50 ± 2.4 $^b$</td>
</tr>
<tr>
<td>DX + CX</td>
<td>53 ± 3.4 $^b$</td>
</tr>
</tbody>
</table>

$^a$ Mean ± S.D. from at least 3 replicate plates.

$^b$ Significantly different ($p < 0.05$) from accumulation in the cells incubated without any additions. Analysis of variance was used.

Distribution of Zinc in Cytosol From Control and DX-treated Cells. As shown in Table 1, whole cells incubated for 18 hr in medium containing DX did not accumulate significantly more total zinc than control cultures. However, the distribution of zinc in the cytosol fraction was altered in DX-treated cells. Sephadex G-75 chromatography of cytosol from control and DX-treated cultures is shown in Chart 3. To account for hormonally induced changes in total protein synthesis and slight
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Chart 2. Exit-exchange of zinc by PC-3 cells. The cells were incubated in 65Zn containing M199 medium with (•) or without (O) 10^-6 M DX for 18 hr. The cultures were washed and reincubated at 37° with fresh nonradioactive M199 plus 5% serum. At given times, cells were washed, and the quantity of 65Zn in whole-cell extracts was determined. Data are expressed as mean percentage (3 replicate plates from 2 separate experiments) of radioactivity remaining relative to that in non-DX-treated cells at 18 hr of culture.

Chart 3. Gel filtration chromatography of cytosol from PC-3 cells incubated for 18 hr with either 65Zn or [35S]cystine. Cultures containing 65Zn contained serum and those containing [35S] did not. Cytosol from control cultures (O) or cultures containing 10^-6 M DX (•) is shown. Fractions were 1 ml. Purified rat liver MT elutes between Fractions 33 to 40 Fractions 16 to 26 are referred to as Peak 1 in the text.

Variations in the cytosol preparations, all analyses and comparisons of specific alterations in zinc distribution were calculated on the basis of the total protein concentration in cytosol preparations. Cytosol from control cells fractionated into 2 major zinc-containing peaks, one representing high-molecular-weight (Fractions 16 to 26) zinc-containing proteins and a lower-molecular-weight zinc-binding fraction that eluted (Fractions 32 to 42) in a fashion identical to the zinc-binding protein, MT (MT-like). The elution profile of DX-treated cell cytosol was identical, except that additional zinc was associated with the lower-molecular-weight fractions. Ion exchange chromatography of this fraction resolved the zinc-binding species into 2 components (Chart 4) which eluted identically with the 2 major forms of MT purified from liver (19).

When cells were incubated with or without DX in medium containing [35S]cystine and 65Zn, 35S eluted with the same fractions as the 65Zn, i.e., those corresponding to the high-molecular-weight proteins and the lower-molecular-weight MT-like fractions, plus a third peak corresponding to small peptides and unincorporated amino acids (Chart 3). Because of the lower specific activity of [35S]cystine in serum-containing medium, there was better incorporation of 35S in cytosol preparations from cells incubated for 18 hr in serum-free medium. Nevertheless, under either condition of incubation, 30 to 40% more [35S]cystine was incorporated into the MT-like peak in DX-treated cultures, indicating there was some de novo protein synthesis.

Chart 4. Purification of MT from cytosol of PC-3 cells cultured with 10^-6 M DX and 65Zn. The cytosol was first chromatographed on Sephadex G-75. Fractions in the region where liver MT is known to elute were pooled and further purified by ion exchange chromatography on DEAE-Sephadex. The column was eluted with 20 to 200 mM linear gradient of Tris-acetate. Results are expressed as cpm 65Zn per fraction (3 ml) (O). — — - - - gradient.

Distribution of Zinc in Cytosol at Various Times. An increase in zinc associated with the MT-like protein in DX-treated cells occurred after 12 hr, which was substantially before a detectable increase in net zinc accumulation. After longer incubation periods, increased amounts of zinc were found in both higher-molecular-weight proteins (Peak 1) and the MT-like peak from control and DX-treated cultures. However, the percentage of increase in zinc content was observed only in the MT-like peak from the DX-treated cells (Chart 5).

Elevation of the zinc concentration of the culture media approximately 10-fold to 100 µM markedly increased the amount of zinc in both Peak 1 and the MT-like peak, but there was a much higher percentage of increase in zinc associated with the latter (Chart 5).

Effect of Other Steroid Hormones and PGE2 on the Distribution of Zinc in Cytosol. Incubation of PC-3 cells for 18 hr in medium containing 5% Bobby calf serum and supplemented with 10^-6 M concentrations of various steroid hormones or PGE2 resulted in changes in the distribution of cytosol zinc as shown in Chart 6. DHT resulted in increased zinc associated with both the high-molecular-weight fractions (Peak 1) and the MT-like peak. When cells were exposed to either 10^-6 M DX or 10^-6 M DHT for 3 hr, rinsed, and incubated 18 hr in media without additional hormone, changes in the zinc distribution were similar to those observed after constant hormone exposure. Hy-
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**DISCUSSION**

The development of neoplasia is a multistage phenomenon in which cells undergo a series of qualitative and quantitative changes which progressively assume a permanent and irreversible character (9). Some biochemical aspects occur long before neoplasia becomes histologically evident. One recognizable biochemical feature of prostatic carcinoma is the low zinc concentration compared with hypertrophied or normal tissue (12, 14). Experiments with animals, as well as clinical observations in humans, have shown that prostatic zinc is under hormonal control. Administration of estrogen or castration decreases normal prostatic zinc levels, whereas administration of testosterone increases the concentration of zinc in the prostate gland (11, 28). Further, reports have demonstrated that the concentration of free zinc in prostatic cells influences the metabolism of testosterone to DHT (33). Since zinc levels are depleted in carcinoma of the prostate, there may be a relationship between zinc and the abnormal testosterone levels. In fact, Habib et al.
(14) reported a marked reduction in DHT concentration when endogenous zinc levels were below 3 µg/g dry weight of human prostatic tissue specimens. This event apparently precedes the accumulation of testosterone which was observed in the malignant gland. The mechanisms by which zinc modulates specific functions in the prostate are unknown.

Little is known of the biochemical forms in which zinc exists in normal prostatic tissue. Reed and Stitch (29) isolated a zinc-binding protein from cytosol fractions prepared from human benign hypertrophic prostates. Further analyses revealed that this was a histidine-rich protein with an empirical molecular weight of 32,000 (17). In further studies, Habib and Stitch (16) isolated a zinc-containing protein from human prostatic tissue which showed affinity for androgens. This protein exhibited a low absorbance at 280 nm, and amino acid analyses demonstrated an absence of aromatic amino acids and a low content of histidine and arginine which is consistent with properties of mammalian MT (19). In the present work with PC-3 cells, the dimorphic migration of the MT-like fraction on DEAE-ion exchange chromatography, inducibility by DX, and inhibition of synthesis by actinomycin D suggest this protein is MT (19). An amino acid analysis would be necessary to establish this point conclusively. Habib (15) has also observed a rapid rise in the zinc concentrations of hyperplastic prostatic tissue following an increase in cadmium content which could reflect a redistribution or increased accumulation of zinc following MT induction by cadmium. Disruption of zinc homeostasis in prostatic tissue by cadmium could interfere with the zinc-androgen interrelationship necessary for normal prostatic function.

The results presented here with the recently established prostatic adenocarcinoma cell line further demonstrate the influence of hormonal control on zinc metabolism. Unlike primary rat hepatocytes (6) or HeLa cells (21), there is a relatively large amount of zinc associated with the MT-like fraction in control cultures. The amount of zinc associated with this fraction was specifically increased by DX, DHT, hydrocortisone, PGE2 while other steroid hormones were inactive. The DX-induced increase in the MT-like protein was due, at least in part, to de novo protein synthesis since there was increased F344sccystine incorporation into the MT-like peak and the increase was sensitive to the protein synthesis inhibitors, CX and actinomycin D. These results suggest that, as in hepatocytes (6), HeLa cells (21), and also liver (4), DX is an inducer of MT synthesis requiring de novo RNA synthesis and probably acts through a glucocorticoid receptor-mediated response (4, 6, 21). However, unlike HeLa cells, changes in the amount of zinc bound to MT was observed in PC-3 cells in response to DHT. These changes were different from those observed with glucocorticoid hormones in that zinc increased in the high-molecular-weight peak (Peak 1) as well as the MT peak. Moreover, it was reported that PC-3 cells do not specifically bind testosterone or DHT in nuclear fractions (20). Thus, it is of considerable interest that DHT causes a major redistribution of cellular zinc in cells which have been reported as androgen insensitive. Since the synthetic glucocorticoid DX is extremely potent relative to other naturally occurring steroid hormones, it is likely that androgens are the major hormonal regulators of zinc metabolism in prostate.

These investigations utilizing PC-3 cells, a human prostatic adenocarcinoma cell line, have demonstrated hormonally induced changes in zinc metabolism in prostatic tissue. They have shown that a MT-like protein may be related to the homeostatic regulation of zinc in prostatic tissue and that both glucocorticoids and male sex hormones can influence zinc accumulation and distribution in these cells.

REFERENCES

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