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ESTROGEN INDUCES SECRETARY PROTEINS IN TRANSFORMED MOUSE LEYDIG CELL IN VIVO, BUT NOT IN VITRO

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Summary: The estrogen-induced proteins were analyzed in one of estrogen responsive mouse Leydig tumors. The incubation of cells freshly prepared from solid tumors with [^{35}S] methionine resulted in the demonstration of estrogen-induced secretary protein with a molecular weight of 34,000. The additional minor estrogen-induced secretary protein (36,000) was also identified. An exposure of these cells to the culture condition for 48 hr caused the loss of their ability to synthesize these secretary proteins even in the presence of estrogen. In contrast, minced tumor tissue was observed to sustain the ability to synthesize these proteins at least for 48 hr. These results would suggest that some cellular arrangement is required for the synthesis of estrogen-induced proteins.

In order to study the molecular mechanism of estrogen-dependent events, the well-defined culture system has been considered to be desirable. In this context, one of major problems is that many cell lines derived from different tumors which are proved to be estrogen-responsive in vivo are found to show no direct stimulatory effect of estrogen on cell proliferation in vitro (1). The lack of in vitro estrogen effects might be explained by the possible role of mesenchymal cells and/or the circulating hormones for elucidating estrogen action (2, 3). Recently, estrogen target cells have been reported to secrete growth stimulators (4). These growth factors effectively stimulate the mitosis of the cultured cells derived from estrogen-responsive tumors. These observations would suggest that the synthesis and/or the secretion of estrogen-dependent proteins are markedly disturbed in the culture condition. To our knowledge, however, this possibility have not been addressed.

Abbreviations: CB-154, ergocriptine; estradiol (E_2), 1,3,5(10)-estratrien-3,17β-diol; FCS, fetal calf serum; TCA, trichlor acetic acid

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One of mouse Leydig cell tumor lines (T 124958) has been found to be estrogen-responsive since the estrogenization of host mice enhances the tumor growth (5). The simultaneous injection of CB-154 did not affect estrogen-induced enhancement of the tumor growth, suggesting that the pituitary hormones, such as prolactin, does not play the major role for this tumor growth. We took advantage of this situation to examine the possible alteration in the secretary behavior of estrogen-induced proteins in response to the in vitro culture conditions.

MATERIALS AND METHODS

The original mouse Leydig cell tumor and host mice (BALB/c strain) were kindly supplied by Dr. R.A.Huseby. One tumor line (T 124958) was recently found to be estrogen-responsive (5). Using this tumor, we established a new subline under the selection pressure of estrogen which shows a marked estrogen-dependence with respect of the tumor growth (6). This new line (T 124958-R) was maintained in mice estrogenized with estradiol (E_2) pellet to be used for the present experiments. This tumor was also found to contain the high level of estrogen receptor (200-300 fmoles/mg cytosol protein)(6).

In order to prepare the dispersed cells, the minced tumor tissue was digested with collagenase (Sigma, type IV) (0.5 mg/ml)-trypsin (Worthington) (0.1 mg/ml) as reported previously (5). These dispersed cells were resuspended in 10% foetal calf serum (FCS)-Eagle's minimum essential medium (MEM) supplemented with 2 mM glutamine and 60 µg/ml kanamycin or methionine-free RPMI 1640. The viability of cells was found to be 75-85%, judged by the trypan blue dye exclusion method. Leydig cell was identified by the nitroblue tetrazolium staining method (7) and observed to be 90-95% in the collected cells. For the long-term culture, cells were plated in 35 sq-mm plastic dishes in 10% FCS-MEM medium containing 10^{-8} M E_2. After 12 passages, the cell cloning was performed by the limited dilution method. The cloning effeciency was found to be 44%. One clone (e-1) was subjected to additional 3 passages before the inoculation into mice. When the in vitro effects of E_2 was examined, FCS was pretreated with dextran coated charcoal (1%) at 37°C for 30 min to remove E_2.

To label the newly synthesized proteins with [^{35}S] methionine, the cells (10^6/well) were plated in 16-mm plastic wells in 1 ml of methionine-free RPMI 1640. In some experiments, the minced tissues (20 mg/well) were used. Labeling was done by adding 28 µCi [^{35}S] methionine (Radiochemical Center Amersham; SA : 800-1,300 Ci/mole). After incubations for the indicated periods of time, the medium was removed and centrifuged for 3 min at 1,000 rpm. The aliquots (0.8 ml) of the supernatants were mixed with 5 µl of bovine serum albumin (20 mg/ml) solution and then subjected to TCA precipitation. After washed twice with 10% TCA and twice with ether:ethanol (1:1) , the pellet was resolved in 60 µl of the electrophoretic sample buffer (0.4%(w/w) sodium dodecyl sulfate (SDS), 2%(v/v) 2-mercaptoethanol, 20%(v/v) glycerol, 0.04%(w/v) bromophenol blue and 125 mM Tris (pH 6.8)), and heated at 100°C for 4 min. After a removal of the medium, the cells were rinsed twice with Hank's solution and resolved in 0.1 ml of the sample buffer. The samples were analyzed by SDS/polyacrylamide gel electrophoresis (8). The slab gels (10% acrylamide) were then impregnated and fluorograms were obtained (9).

RESULTS AND DISCUSSION

The estrogenization of host mice with the subcutaneous implantation of E_2 pellet markedly enhanced the tumor growth (6). In order to examine the possi-

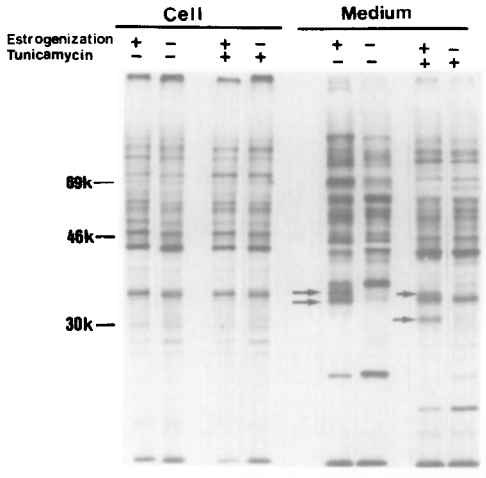

Fig. 1 Identification of estrogen-induced secretary proteins
The tumors were subcutaneously transplanted into mice estrogenized with the E_2 pellet (6). The estrogenization of one mouse was discontinued by a removal of the E_2 pellet on day 14 after transplantation. Both estrogenized and nonestrogenized mice were sacrificed on day 24. The dispersed cells were incubated with [^{35}S] methionine for 24 hr in the presence or the absence of tunicamycin. The newly synthesized proteins were analyzed as described in Materials and Methods. The molecular weights were determined by the mobilities of [^{14}C] labeled protein markers (Radiochemical Center Amersham). The arrows indicate the estrogen-induced secretary proteins.

ble correlation of estrogen-enhanced cell proliferation with protein markers, estrogen-induced proteins were studied. The preliminary studies revealed that the typical estrogen marker such as progesterone receptor was not able to be identified. This information in turn led us to characterize the proteins newly synthesized in cells with or without estrogenic stimuli. As shown in Fig. 1, in vivo E_2 stimulation of the tumor cells resulted in an appearance of the secretary protein with a molecular weight of 34,000 without any detectable estrogen-induced intracellular proteins. The inclusion of tunicamycin (10 μg/ml) in the medium was observed to reduce the molecular weight of this estrogen-induced secretary proteins to 31,000, suggesting that this secretary protein is N-glycosilated (10). Another secretary protein with a molecular weight of 36,000 was also stimulated by in vivo estrogen treatment. However, some experiments failed to demonstrate the estrogen-dependent synthesis of this 36 K protein under the condition used for this experiment (see below). The effect of the preculture on these estrogen-induced secretary proteins was next examined (Fig. 2). The 2 hr exposure of these cells to [^{35}S] methionine

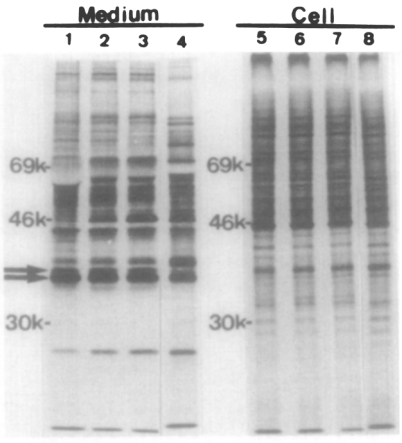

Fig. 2 Rapid disappearance of 36 K protein after plating.
The cells were prepared from the solid tumor stimulated with E_2 in vivo, and exposed to [^{35}S] methionine for 2 hr after the brief preculture for the various periods. The proteins labeled were analyzed by electrophoresis lane 1-4, secretary proteins; lane 5-8 intracellular proteins; lane 1 and 5, labeling immediately after plating; lane 2 and 6, labeling after a 2 hr-preculture; lane 3 and 7, labeling after a 4 hr-preculture; lane 4 and 8, labeling after a 6 hr-preculture.

after brief preincubation was employed to label newly-synthesized proteins. The 36 K protein was found to be rapidly decreased with the increased preincubation periods. The delayed addition of [^{35}S] methionine 6 hr after plating cells into dishes resulted in the failure of the detection of 36 K protein on the fluorography. This rapid cessation of the synthesis of 36 K protein might be related to the failure of the reproducible demonstration of this protein. The 48 hr-preincubation of the dispersed cells in 10% FCS-MEM+10^{-8}M E_2 (Fig3), followed by labeling with [^{35}S] methionine for 24 hr, resulted in a marked decrease of 34 K secretary protein. In addition, the established cell line also failed to show estrogen-dependent secretion of 34 K and 36 K proteins (Fig.4). The simultaneous addition of HCG (10 ng/ml), insulin (1 µg/ml), dexamethasone (10^{-8} M) and transferrin (20 µg/ml) into the medium did not resulted in the sustained ability of the cell to secrete 34 K and/or 36 K proteins. (data not shown) In order to obtain some clue for this event, the minced tissue was preincubated in 10% FCS-MEM + 10^{-8} M E_2 for 48 hr, and then labeled with [^{35}S] methionine. As illustrated in Fig. 3, the 34 K protein was identified as a major secretary protein. This result might raise the interesting possibility

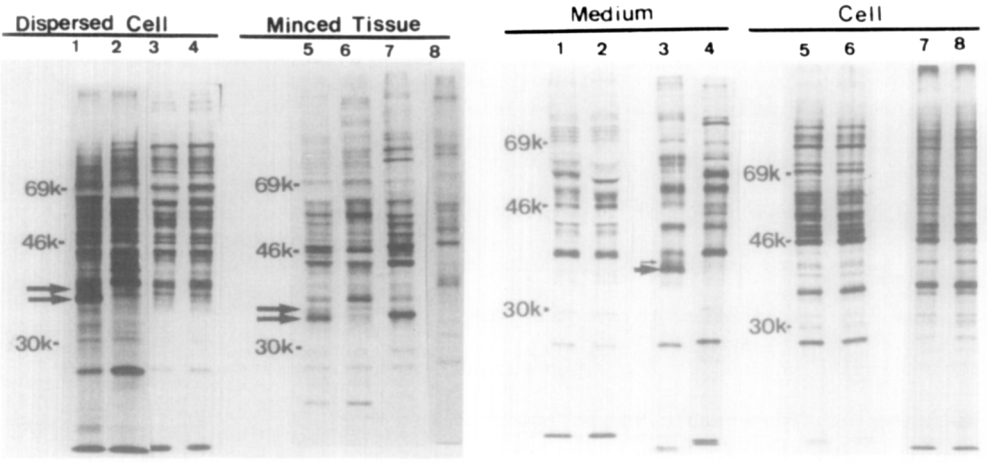

Fig. 3 Difference in the sustained ability to secrete estrogen induced proteins between the dispersed cells and the minced tissue.
The dispersed cells or the minced tissue was prepared from the solid tumors stimulated or nonstimulated with E_2 as described in the legend of Fig. 1, and then incubated with [^{35}S] methionine for 48 hr after precultures in 10% FCS-10^{-8} M E_2-MEM medium. The secretary proteins were electrophorezed.
lane 1-4, dispersed cells; lane 5-8, minced tissue; lane 1,3,5 and 7, tumor stimulated with E_2 in vivo; lane 2,4,6 and 8, nonstimulated tumor; lane 1,2,5 and 6, labeling immediately after plating; lane 3,4,7 and 8, labeling after 2 days-preculture.
The arrow indicate estrogen-induced secretary protein.

Fig. 4 Effects of in vivo or in vitro estrogen stimulation on the ability of the cloned cell (e-1) to secrete the proteins.
The cloned cell was cultured in 10% charcoal treated FCS-MEM medium with (lane 1 and 5) or without (lane 2 and 6) 10^{-8} M E_2 for 14 days followed by labeling with [^{35}S] methionine. The cloned cells (5×10^5) were also subcutaneously inoculation into estrogenized (lane 3 and 7) or nonestrogenized (lane 4 and 8) mice. The solid tumor (ca 1.5 gr) were formed approximately one month (estrogenized mice) or two month (nonestrogenized mice) later after inoculation. The dispersed cells were prepared from these tumors and immediately used for protein labeling. The secretary (lane 1-4) and intracellular (lane 5-8) proteins were electrophorezed. The arrows indicate the estrogen-induced proteins.

that some special arrangement of malignant cells is required to synthesize estrogen-induced secretary proteins. An important role of cell shape for elucidation of in vitro hormone responsiveness has been proposed in mammary epithelial cell (11). The altenative explanation for this discrepancy between the minced tissue and the dispersed cells might be that some important factor for the sustained estrogen action, which is associated with the cell membrane in vivo, was eliminated during preparation of the disperced cells with enzymes. The addition of the serum from estrogenized mice into the culture medium at a final concentration of 10% was without effect, suggesting that the involvement

of the circulating hormones in the experimental results is negligible (data not shown). Nevertheless, the possibility that the genes coding these estrogen-dependent secretary proteins were deleted during in vitro manipulation seems to be unlikely, since the subcutaneous inoculation of the cloned cells resulted in a reappearance of 34 K protein as a major secretary proteins (Fig. 4). The cells were usually labeled with [^{35}S] methionine for the relatively long time (24 hr) in order to see the possible accumulation of some secretary proteins inside the cell. The disappearance of estrogen-induced secretary proteins without detectable accumulation of intracellular proteins (Fig.2 and 4) would suggest that the biosynthic, but not secretary, process of these estrogen-induced proteins was disturbed by an exposure of these cells to the in vitro culture condition.

Although some cultured cells have been reported to be able to secrete the proteins in response to estrogenic stimuli (12, 13), many cells derived from estrogen-responsive tumors have observed to fail to response to in vitro estrogen stimulation. These well-known, but unresolved, phenomena might be related to the experimental results presented in this communication.

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