Response to Estrogen by the Human Mammary Carcinoma Cell Line CAMA-1

Benjamin S. Leung, Shehla Qureshi, and Jonas S. Leung

Department of Obstetrics and Gynecology, University of Minnesota Medical School, Minneapolis, Minnesota 55455

ABSTRACT

CAMA-1 cells, isolated from malignant pleural effusion, are grown in long-term cultures as monolayers. The rate of growth is dependent upon fetal bovine serum and estradiol. These cells also exhibit a dose response to added 17β-estradiol with respect to the incorporation of radiolabeled thymidine into cells. The uptake is increased at low levels of estradiol and decreased at pharmacological levels of estradiol. The uptake of uridine and leucine is also stimulated by estradiol in a dose-related manner. Induction of precursor uptake is not observable until cells have been exposed to estradiol for approximately 10 hr or longer. Cells plated for different periods in steroid-stripped serum remain sensitive to estrogenic stimulation and show similar lag time in response. Estrogenic effect is not noticeable in the absence of serum. Addition of prolactin can partially restore estrogenic stimulation of thymidine uptake in serum-free medium. Like other estrogen target tissues, these cells contain cytoplasmic and nuclear estrogen receptors. These results demonstrate that the CAMA-1 cell line is estrogen dependent and that these cells may provide a promising model for the in vitro investigation of the mode of estrogen action in human breast cancer.

INTRODUCTION

It is known that the growth of breast cancer cells is influenced by many hormones (13). Estrogen and prolactin are important in stimulating growth of mammary tumors in rodents (2, 3, 11, 15, 19, 22, 27). Recent reports (16-18, 21) demonstrated that the growth of an established cell line (MCF-7, isolated from pleural fluid of a breast cancer patient) was stimulated by low levels of estrogen and inhibited by antiestrogen or high levels of estrogen.

In this report, we present results of another established cell line (CAMA-1) which responds to estrogen in precursor uptake and cell growth.

MATERIALS AND METHODS

Cells and Tissue Culture. CAMA-1 is a human breast cancer cell line originating from the malignant pleural effusion of a postmenopausal woman with adenocarcinoma of the breast (4). These cells were grown in T-75 flasks as monolayer cultures in Eagle’s minimum essential medium originating from the malignant pleural effusion of a postmenopausal and cell growth.

25% FBS (Grand Island Biological Co., Grand Island, N. Y.). Culture conditions, harvesting of cells for experiments, and seeding new passages have been described previously (30).

Precursor Incorporation. Cells growing in the log phase were plated in T-25 flasks at a density of 5 x 10^6 cells/flask. They were grown in minimum essential medium, supplemented with predescribed constituents and the corresponding proportions of FBS or DCFBS [serum treated with dextran-coated charcoal to remove free steroids (30)]. In experiments in which the effect of 17β-estradiol was studied, it was dissolved in ethanol before addition to the medium so that the final ethanol concentration was 0.1% or less. After incubating for the intended period of time, cells were pulsed with tritiated precursors (0.5 μCi), usually conducted for 2 hr, unless otherwise stated. Following aspiration of culture medium, cells were harvested by washing with ice-cold 0.9% NaCl solution, suspending in 0.025% trypsin-EDTA, and collecting on glass fiber filters (Reeve Angel 934 AH) with a 22-hole vacuum-manifold. After 2 or more washes with cold 0.9% NaCl solution, the filters containing cells were put into scintillation vials. The cells were digested by Protosol (New England Nuclear, Boston, Mass.) at 60° for 0.5 hr, and 10 ml Omnifluor-toluene (New England Nuclear) were added. Radioactivity was determined by a Beckman 350 scintillation system with an efficiency of 35 to 40% for tritium.

RESULTS

When CAMA-1 cells were cultured in a system not supplemented with serum, cell proliferation and monolayer formation were very poor, probably because there are abundant nutrients and constituents in the serum that are essential for cell growth. These substances include various steroid and peptide hormones. As shown in Chart 1, the serum requirement for cell growth is evident. In the presence of estradiol (1 nm) and different proportions of FBS, the initial rate of cell proliferation among these cultures was not different (Chart 1A). However, the total number of cells harvested on Day 7 from flasks with 10% or less FBS was much lower than with higher amounts. This effect of serum was amplified on Day 10, because cells grown in less than 20% FBS went into stationary phase by Day 7, contributing to a much lower cell yield at harvest than when cells were grown in 25% FBS. These results show that a depletion of essential nutrients from low serum cultures occurred following the fourth day of plating. When cells were grown in serum treated with dextran-coated charcoal to remove free steroids and other materials that adsorbed to the charcoal surface, the rate of cell proliferation was much lower (Chart 1B). With the addition of estrogen, the rate of cell growth was partially restored. This effect of estradiol was noticeable with other proportions of DCFBS added (result not shown). Apparently, other constituents of serum that were removed by dextran-coated charcoal were also important for sustaining cell growth.

To further test the requirement of estradiol for cell proliferation, CAMA-1 cells were cultured in medium supplemented with 25% DCFBS (Chart 2). While cell yield in 25% FBS remained fairly constant at the end of each 7-day culture, from 2 separate experiments, cells grown in 25% DCFBS showed a constant and continuous decrease in cell number at harvest. It

1 Supported in part by National Cancer Institute Research Grant 1R01-CA 25998. Part of this work was presented at the 60th Annual Meeting of the Endocrine Society, 1978 (14).

2 To whom requests for reprints should be addressed, at Box 395, Mayo Memorial Building, 420 Delaware Street S.E., Minneapolis, Minn. 55455.

3 The abbreviations used are: FBS, fetal bovine serum; DCFBS, dextran-coated charcoal-treated fetal bovine serum; DThd, ['H]thymidine; ER, estrogen receptor.

Received August 31, 1981; accepted August 5, 1982.
Estrogen Response in Breast Cancer CAMA-1 Cells

Chart 1. A. effect of FBS on the rate of CAMA-1 cell growth in the presence of 1 nM estradiol and different amounts of FBS (as percentages). Points, close means of triplicate determinations. B. cell growth in 15% DCFBS with (○) or without (□) 1.0 nM estradiol (E). Growth curve in 15% FBS was taken from data in A as a reference. Each value is the mean of triplicate determinations.

Chart 2. Effect of DCFBS on the maintenance of cell growth in culture. Each culture was plated with 10^5 cells in a T-75 flask, as described in “Materials and Methods,” either in 25% FBS or 25% DCFBS without the addition of estradiol. During harvest on Day 7, cells were suspended in 5 ml medium; 1-ml aliquot was used for determination of cell number, another aliquot was plated as a new passage. Data points (○) and (●) for DCFBS cultures were from 2 separate experiments; (●) and (●) values were from the same experiment. Cell numbers are means of triplicates determined during harvest.

Chart 3. Effect of estradiol on cell proliferation in 10% DCFBS. CAMA-1 cells were cultured in T-25 flasks, and on Day 7, cell numbers were determined and were expressed as a percentage of the number of cells plated (50,000 cells, 100%). A. no estradiol added during plating for each new passage (open bar). At the beginning of the fourth passage, cells from cultures without estradiol were supplemented with 1.0 nM estradiol (stippled bar). B. 1.0 nM estradiol added at time of plating of each new passage (hatched bar). Each value is the close mean of triplicate determinations.

was observed that many viable, unattached cells were floating in the media during prolonged culture in DCFBS. These floaters, however, accounted for only a fraction of the cell loss. It is known that complete removal of serum estradiol by dextrancoated charcoal adsorption is seldom complete; nevertheless, the unadsorbed estradiol concentration must be too low for the maintenance of cell growth following the first passage. In order to assure that the removal of estradiol at least in part contributed to cell death, an estrogen rescue experiment was conducted (Chart 3). To minimize the effect of any remnant estradiol in the serum, a 10% DCFBS was selected for continuous passages of the cells, either in the presence or absence of 1 nM estradiol. Cells grown in DCFBS, with or without estradiol, stopped proliferating after the first passage. A 40% decline in cell number was noted following the second passage in 10% DCFBS without estradiol (Chart 3A). Although estrogen could not stimulate cell proliferation in the second passage (Chart 3B), it prevented the decline in cell number, and a partial restoration of cell growth was noted by the fourth passage. If estrogen was added at the beginning of the fourth passage to chronically estrogen-deprived cells (Chart 3A), a prompt doubling of cell number was noted. These results demonstrate that estrogen is one of the basic elements necessary for CAMA-1 cell maintenance and growth in culture.

It is desirable to have a simple assay which can reflect the incorporation of thymidine into DNA molecules. First, the methods of dThd uptake into whole cells was compared to the conventional method of 10% trichloroacetic acid precipitation. Following pulsing for 0.25 to 4 hr (Chart 4A), radioactivity uptake into whole cells parallels that of trichloroacetic acid-insoluble materials. These 2 methods were further evaluated in another experiment by determining the effect of estradiol upon dThd uptake (Chart 4B). Similar to results shown in Chart 4A, whether in the presence or the absence of estradiol, radioac-
Chart 4. A, whole-cell dThd uptake versus trichloroacetic acid precipitation. Replicate nonconfluent monolayer cultures of CAMA-1 cells were labeled by adding 0.25 μCi dThd to plates for the indicated time. Cultures were terminated by removal of the labeled medium, one rinse with 0.9% NaCl solution, and detachment with trypsin. O, cells filtered, dried, and counted in Budgetsolve (New England Nuclear), and counted in Omnifluor-toluene (New England Nuclear). A, cells were precipitated with 10% trichloroacetic acid and washed twice, and the precipitates were digested with Protosol and counted in Omnifluor-toluene. In all cases, dThd uptake is linear with a slope of 1.0. B, effect of estradiol on whole-cell dThd uptake versus trichloroacetic acid precipitation. Replicate nonconfluent monolayer cultures of CAMA-1 cells growing for 48 hr either in the presence of estradiol (1.0 nm) or in its absence. Cells were pulse labeled with 0.25 μCi dThd for the time indicated and were harvested as described in A; one set was filtered, dried, and counted in Budgetsolve (O), while the other set was precipitated by trichloroacetic acid (△). Estradiol-induced dThd uptake was expressed as a percentage over controls without estradiol (100%).

The relationship between cellular dThd uptake and cell proliferation during a 7-day culture period was evaluated (Chart 5). The level of dThd uptake into whole cells again parallels the cell yield derived from Coulter counting. Collectively, these results show that 2-hr pulse labeling of whole cells can be used as a reliable index for determination of dThd incorporation into DNA molecules.

The duration required for estrogen to exert an effect on dThd uptake was evaluated (Chart 6). In this experiment, CAMA-1 cells were incubated for 24 hr in 25% DCFBS prior to the addition of estradiol. A slight decline of dThd uptake, probably due to cell death or progression of cells from S phase to G2 phase, was observed during the first 8 hr of estrogen exposure. By 16 hr, a more than 2-fold increase in dThd uptake was reached, and remained at this maximal level up to 24 hr following the addition of estrogen. When cells were cultured for 48 hr in the presence of estradiol, added during plating, the level of dThd was no different than the maximal level induced by estradiol after a 16-hr exposure. In contrast, control cells incubated for 48 hr without added estradiol had dThd radioactivity equivalent to uptake in cells exposed to estradiol for 2 hr. These results show that estradiol has an early effect on CAMA-1 cells in the uptake of dThd. The observed lag time in this experiment may represent the duration of the G1 phase under estrogen environment. It appears that the transition of S phase to G2 occurred from 8 to 16 hr following estrogen exposure.

To rule out the possibility that the observed estrogen stimulation may be an artifact, the sensitivity of cells to estrogen was examined in DCFBS following plating for 24 hr, 48 hr, or 7 days (Chart 7). In each case, estradiol induced a marked increase in dThd uptake, and the induction invariably commenced at 10 to 12 hr following the addition of estradiol.

However, the pattern of the induction rate in each case was not the same. Control cells without added estradiol uniformly showed a much reduced rate of dThd uptake; only slight increase was noted during the period between 10 and 16 hr following the addition of vehicle as compared with estradiol-treated cells. Under the present culture condition, cell number at Day 7 was approximately double that at Day 2. A 20-fold increase in dThd uptake in both control and estradiol-treated cultures in comparison to respective cultures at 24 and 48 hr suggests that most of the cells at Day 7 were in the G2 phase. The addition of dThd was able to initiate a traverse of the cell cycle. This phenomenon was later on confirmed by cell cycle analysis. Virtually no S-phase population remained on Day 7 while 80 to 90% of the cells were in the G2 phase. The stimulation of dThd uptake in estrogen-treated cells may then represent a faster transition from G2 to S phase. These results further indicate that estradiol is needed to promote a faster progression from G2 to S phase in DCFBS environment.
dThd, the effect of estrogen in stimulating a continuous uptake of dThd into cells during a 7-day culture period was examined (Chart 8). In the medium supplemented with 25% FBS, maximal incorporation of dThd added during plating was reached by 48 hr; this level of dThd radioactivity was maintained in subsequent days by CAMA-1 cells, possibly indicating that the rate of S-phase cell formation was balanced by the rate of cell death. In cultures where cells were grown in media supplemented with 10 nm estradiol in 25% DCFBS, a much higher rate of dThd uptake was observed during the first 48 hr. The subsequent rate of cell death, however, was more rapid than the rate of cell proliferation. Certain growth nutrients in FBS that were removed by dextran-coated charcoal might have contributed to the faster cell death. If estrogen effect were to increase the pool size of dThd, this effect would be most noticeable during the initial addition of dThd, and as equilibrium was reached, this effect would be greatly reduced. From our results, estrogen exerted a maximal rate of dThd uptake during the first 24 hr, and a maximal level of uptake was reached at 48 hr following the addition of dThd. It seemed unlikely that the late induction by estrogen can be accounted for by the increase in pool size. These results may best be explained by the ultimate increase in the incorporation of dThd into DNA molecules, either through DNA repair or through new synthesis. The finding from continuous labeling experiments is consistent with results of pulse labeling (Charts 6 and 7), in which maximal rate of estrogen stimulation also occurred within 24 hr after the addition of estradiol.

The degree of induction of dThd uptake in DCFBS conditions is dose dependent on estrogen. Almost a linear logarithmic increase was observed from 10 pm to 10 nm (Chart 9). Although sensitivity to estrogen varies with different passages of cells and the amount of added DCFBS, this dose-dependent relationship can be demonstrated repeatedly. The lowest level of estradiol that is effective was usually at 10 pm. Following its maximal induction, usually at 1.0 to 10 nm, depending on incubation conditions and passages of cells, a reverse trend of dThd uptake set in when higher concentrations of estradiol were used. At levels above 10 µm estradiol, an inhibition of dThd uptake to levels below that of control cells was often encountered (data not shown).
In the absence of serum, CAMA-1 cells attached poorly to the surface of culture flasks, and cell death occurred precipitously 3 days following plating. A 10% serum substitute (12), which contains constituents such as vitamins, fatty acids, phospholipids, amino acids, insulin, thyroxine, hydrocortisone, and cholesterol, can maintain CAMA-1 cell growth. In fact, the rate of cell proliferation in 10% serum substitute plus 1% DCFBS is almost equivalent to that in 25% FBS during a 7-day culture period (12). However, when dThd uptake was evaluated in a

Similar to dThd uptake, estrogen also stimulated the uptake of [3H]uridine and [3H]leucine into cells as compared to controls without added estradiol (Chart 9). The inductions again were dose dependent. The time course of estrogen induction of [3H] uridine is shown in Chart 10. No stimulation by estradiol was observed at 8 hr, but significant stimulation was noted at 16- and 24-hr exposures to estradiol added during plating.

In the absence of serum...
Estrogen Response in Breast Cancer CAMA-1 Cells

were compared to control cells. Nevertheless, high levels of for 1 hr at 4°C with intermittent vortexing. KCl extract was used as the source for crude nuclear pellet with 0.4 M KCl Tris-HCl-glycerol-EDTA-dithiothreitol buffer was included in gradient buffers. Nuclear ER (ERn) (top) was extracted from total binding determinations (•); 100-fold diethylstilbestrol was used as competitor for nonspecific binding determination (O); and sodium molybdate (20 mM) incubation was conducted for 4 hr at 0–4°C; 2.5 nM [3H]estradiol was used for gradient technique were as described previously (15), with the exception that EDTA-dithiothreitol buffer containing 20 mM sodium molybdate. Methods of scraping, homogenized with a glass-Teflon homogenizer in Tris-HCl-glycerol-

At Day 7, cells from 8 T-75 flasks (5 × 10⁶ cells/flask) were harvested by scraping, homogenized with a glass-Teflon homogenizer in Tris-HCl-glycerol-

Chart 13. Sucrose gradient profiles of estrogen receptors of CAMA-1 cells. At Day 7, cells from 8 T-75 flasks (5 × 10⁶ cells/flask) were harvested by scraping, homogenized with a glass-Teflon homogenizer in Tris-HCl-glycerol-

DECEMBER 1982 5065

Estrogen Response in Breast Cancer CAMA-1 Cells

...stromal serum substitute (Chart 11), estradiol could not elicit a stimulatory effect. In many other experiments conducted on dThd uptake, estradiol had no effect and only occasionally showed a marginal stimulation when estrogen-treated cells were compared to control cells. Nevertheless, high levels of estradiol always were inhibitory. The level of estradiol that was necessary to achieve inhibition was usually about 10-fold lower than that required under DCFBS conditions. Interestingly, the addition of prolactin reestablished, at least partially, the stimulatory effect and reduced the inhibitory effect of estradiol. In this regard, it may be speculated that prolactin might be involved in the enhancement of cell cycle transverse elicited by estradiol.

The effect of serum in a nutritionally adequate environment (i.e., 10% serum substitute) was studied, and the time course of dThd uptake due to different proportions of DCFBS in 10% serum substitute and 1 nm estradiol was determined (Chart 12). With 1% DCFBS added, the rate of dThd uptake was low and changed only slightly from 8 to 12 hr following plating. A 50% increase in dThd uptake was observed during the same period in a 5% DCFBS condition. Further increase in dThd uptake was noted with increased proportions of added DCFBS. Results from this experiment indicate that factors promoting a more rapid rate of cell cycle traverse, for example from G0 to S phase, are present in the serum. The relationship of estradiol to these factors, however, requires further experimentation.

It is known that estrogen target tissues contain ER. CAMA-1 cells contain cytoplasmic ER which sediment at the 8S region of a sucrose gradient (Chart 13, bottom). Most of the ER complexes were present in the crude nuclear fraction and could be extracted by a high-ionic-strength buffer, yielding a 4S peak (Chart 13, top). The ER peaks were abolished by an excessive amount of diethylstilbestrol in the incubation mixture.

Besides stimulating precursor uptake and cell growth of CAMA-1, estrogen also induced progesterone receptor biosynthesis (30). Tamoxifen and nafoxidine inhibited this process (29).

DISCUSSION

Since the successful culture of an estrogen-responsive cell line (MCF-7) in long-term culture (25), many studies have been conducted to investigate the role of hormones in this cell line (3, 5, 9, 10, 16–18, 21, 28). Other established human breast cancer cell lines that are estrogen sensitive have also been reported (1, 9). These cells, similar to other estrogen target tissues, contain cytoplasmic or nuclear ERs (7, 9, 16). In this report, we present findings of another established cell line of human origin that responds to estrogen in culture. Results presented in this communication show that CAMA-1 cells depend on estrogen for growth and have the characteristics of an estrogen-dependent cell line. When CAMA-1 cells are grown in DCFBS, cell proliferation is markedly reduced. Chronic insufficiency of estrogen added to cultures also leads to an apparent loss of response of CAMA-1 cells to estrogen-induced dThd uptake (30).

Several investigations have pointed out that estrogen-induced cell proliferation may be mediated through estrogen-induced proteins (20, 23, 24). When CAMA-1 cultures are not supplemented with serum, estrogen cannot elicit a stimulatory effect on dThd uptake. From data presented herein and elsewhere (12), the lack of estrogenic stimulation of dThd uptake in serum substitute appears to be from factors other than nutritional, since CAMA-1 cells proliferate as well in serum substitute as in serum (12). Our results demonstrate that serum factors or proteins may be necessary for estrogen-induced growth in vitro. It remains to be tested whether these substances are related to the estrogen-induced proteins (23, 24) that were described to mediate estrogen action in vivo.

The biphasic response of CAMA-1 cells to estrogen-induced
dThd uptake has been reported by others for MCF-7 cell line (16). It is tempting to relate this characteristic response of cells in vitro to clinical trials, where low levels of estrogen exacerbate breast cancer in patients, while high levels often provide beneficial palliation. However, high levels of estradiol in vitro may simply exert a nonspecific cytotoxic effect upon culture cells. Further experimentation is needed to demonstrate whether or not this inhibitory effect is specific to estrogen-sensitive cells.

ERs in CAMA-1 cells are largely located in the nucleus. These results are consistent with findings reported for other cell lines (7, 9). This phenomenon, however, is different from normal estrogen target tissues such as the uterus, where more than 70% of receptors are present in the cytoplasm. Similarly, human breast cancers also contain high levels of cytoplasmic receptors (11, 19). These results seem to indicate that culture conditions or serum factors might have altered the distribution of ER. It was suggested (8) that the level of nuclear receptors in cultured cells is related to estrogen induction of progestosterone receptors. Although CAMA-1 cells also exhibit a dose response to estrogen induction of progestosterone receptor (30), the quantitative relationship of nuclear receptor and estrogen-induced progestosterone receptor and other biological responses requires further experimentation.

We have demonstrated that estrogen not only stimulates proliferation of CAMA-1 cells, it also stimulates cellular events such as uptake of dThd, [3H]leucine, and [3H]uridine, and the induction of progesterone receptors (30). In other studies, we have shown that the incorporation of oleoyl-CoA into phosphatidylinositols and phosphatidylcholines are also dependent upon estrogen concentration (26). In addition to estrogen, CAMA-1 cells respond to other hormones such as prolactin, progesterone, insulin (12), glucocorticoid, and androgen (6) in culture. Estrogen-induced cellular events such as protein synthesis (28) and progesterone receptor production (8, 9) have also been reported for the MCF-7 cell line. Specific estrogen-induced proteins in CAMA-1 cells are being actively investigated in our laboratory.

In conclusion, CAMA-1 cell line has the unique characteristics of an estrogen-dependent tissue. It may provide a good alternative model for the elucidation of estrogen action in cell proliferation of human breast cancer. The feasibility of using this cell line to understand the interaction of prolactin, estrogen, and progesterone in breast cancer is encouraging (12), and investigations related to the interaction of these hormones in CAMA-1 cells are in progress. The effects of these hormones on CAMA-1 cells in nude mice remain to be explored.

ACKNOWLEDGMENTS

We thank Dr. J. Fogh of Sloan-Kettering Institute for Cancer Research for the generous gift of CAMA-1 cell line, the National Institute of Arthritis, Metabolism and Digestive Diseases for the gift of ovine prolactin, and Anne Potter for excellent technical assistance.

REFERENCES

Response to Estrogen by the Human Mammary Carcinoma Cell Line CAMA-1

Benjamin S. Leung, Shehla Qureshi and Jonas S. Leung


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/42/12/5060

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.