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ESTRADIOL INDUCED PROTEINS IN THE MCF_7 HUMAN BREAST CANCER CELL LINE

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MCF_7 cells were cultured with steroids, labelled with (^{35}S)-methionine and the secreted and intracellular proteins were examined by one and two dimensional gel electrophoresis. Estradiol (0.1 nM) increased the synthesis of some of the secreted proteins ; the induction of a protein of molecular weight 46,000 daltons being the most dramatic. The 46,000 daltons secreted protein was heterogeneous with respect to molecular weight and isoelectric point. The antiestrogen Tamoxifen did not stimulate the synthesis of any of the estrogen induced proteins, but completely inhibited the induction by estradiol. The effect of estradiol on internal proteins was much more subtle ; only 3 proteins out of about 250 were stimulated. The functions of these Proteins are unknown, however they appear to be good markers for studying the mechanism of action of estrogens and antiestrogens in breast cancer and might be related to the control of cell proliferation by estrogen.

Estrogens stimulate the growth of about one third of human breast cancers. Knowledge of the mechanism by which estrogens are acting would not only assist in predicting estrogen responsiveness of tumours (1) but might also suggest better treatment of breast cancer. To study this mechanism in human breast cancer, a good in vitro system with specific probes for estrogen action is required. The MCF_7 human breast cancer cell line has been recently proposed (2) as such a model system. This cell line which was established from a pleural effusion in a patient with metastatic breast cancer (3) contains estrogen receptor. Its growth is inhibited by antiestrogens and appears to be stimulated by estradiol (2). The progesterone receptor is the only protein having been shown to be induced by estrogen in this cell line

Abbreviations used: DEM : Dulbecco's modified Eagles Medium; FCS : Fetal calf serum; TCA : Trichoroacetic acid; SDS : Sodium dodecyl sulphate; Tamoxifen (ICI 46,474) : trans-1(p-dimethylamino ethoxyphenyl)-1,2-diphenyl but-1-ene.

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and it has been proposed as a marker for estrogen responsiveness in human breast cancer (4). However, the usefulness of the progesterone receptor as a marker for estrogen responsiveness is questioned (5). In the hope of finding better markers for hormone responsiveness and better probes for studying estrogen action, we have compared the (^{35}S)-methionine labelled proteins in MCF_7 human breast cancer cells cultured with or without estradiol using one (6) and two (7) dimensional electrophoresis. We show that estradiol but not the antiestrogen tamoxifen, increases specifically the synthesis of several secreted and intracellular proteins.

MATERIAL AND METHODS

Cell culture : MCF_7 cells obtained from Dr M. Lippman (National Cancer Institute, Bethesda, Maryland) and Dr M. Rich (Michigan Cancer Foundation, Detroit) were maintained in monolayer-culture in DEM containing 10 % FCS, streptomycin and penicillin (250 units/ml) and insulin (0.6 µg/ml). They were trypsinised and plated out in 0.8 cm diameter microwells (20,000 cells/well) in 50 µl DEM containing 10 % FCS treated with dextran coated charcoal (2). The cells were grown for two days in the same medium, in order to withdraw cells from estrogen present in FCS. They were then grown for two further days in the same medium with or without steroids or tamoxifen. The media were changed every day both before and during hormone treatment. The MCF_7 cells were cultured according to the standards of biological safety, issued by the National Cancer Institute (Bethesda).

Labelling and analysis of proteins : After hormone treatment the cells were labelled in 50 µl of Eagles minimum essential medium containing one tenth the normal concentration of methionine plus 200 µCi/ml of (^{35}S)-methionine (CEA-France, specific activity : 860 Ci/mmole) for 5 h. Incorporation of (^{35}S)-methionine was linear throughout the 5 h labelling period. The secreted and internal proteins were analysed in the culture medium and lysed cells respectively. The medium was collected and centrifuged at 10,000 g for 4 min. Cells were dissolved in 50 µl of lysis buffer (6) containing 0.5 % SDS. Incorporation of (^{35}S)-methionine was measured in 20 µl medium or 5 µl of lysed cells. The aliquots were mixed with 100 µl of bovine serum albumin solution (0.5 mg/ml) and then precipitated with 1 ml 10 % TCA for 30 min at 0-4°C. The precipitates were collected on glassfiber filters (Whatman GFC) and washed extensively with 5 % TCA containing 5 mM unlabelled methionine. The filters were then dried at 100°C for 6 h and counted in 5 ml scintillation fluid with an efficiency of ≃80 %. For SDS gel electrophoresis, 20 µl of medium or 100,000 cpm of cellular proteins were mixed with an equal volume of buffer containing β-mercaptoethanol (10 %), SDS (4 %), glycerol (20 %), Tris (0.125 M, pH 6.8), EDTA (40 mM) and bromophenol blue (0.01 %). Samples were then heated at 100°C for 2 min and analysed on a 15 % acrylamide gel with 3 % stacking gel containing 0.1 % SDS (5). The two- dimensional electrophoresis was as described by O'Farrell (6) except that isoelectric focussing

gels were 2 mm in diameter and SDS gel electrophoresis was in 1 mm thick slab gels. Either 50 µl of medium (lyophilised and redissolved in 10 µl of lysis buffer) or 300,000 cpm of cellular proteins were analysed. Both one and two dimensional gels were processed for fluorography (6) and exposed to Kodak XR 1 film (prefogged to an absorbance of 0.15). The molecular weights of the major proteins were estimated by their mobilities relative to protein markers.

RESULTS

Cells were incubated with or without estradiol and tamoxifen for 2 days and the secreted and cellular proteins were then analysed separately using one and two dimensional electrophoresis as described in the Methods. Fig. 1 shows polyacrylamide gels of the secreted proteins. At least 15 proteins were visuali-

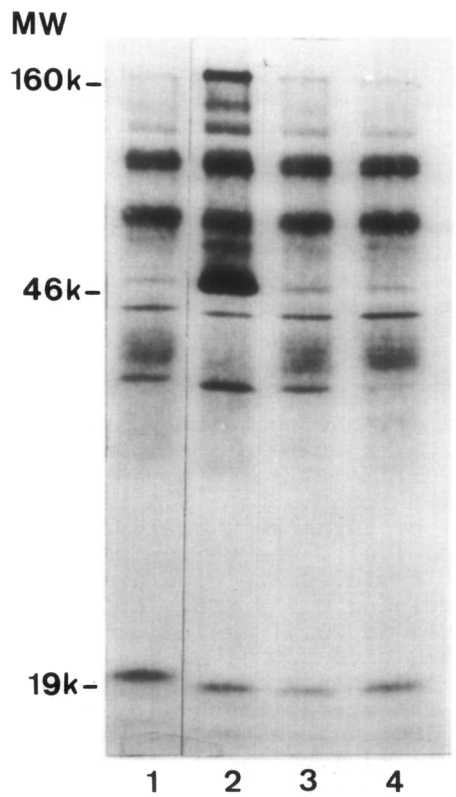

Figure 1 : Effect of estradiol and tamoxifen on the synthesis of secreted proteins in MCF_7 cells : Fluorogram of secreted proteins labelled by (^{35}S)-methionine and separated by polyacrylamide gel electrophoresis. The cells were treated for 2 days in medium containing 10 % FCS treated with dextran coated charcoal alone (tracks 1), the same medium plus estradiol 0.1 nM (tracks 2) or tamoxifen 1 µM (tracks 3) or estradiol + tamoxifen 1 µM (tracks 4).

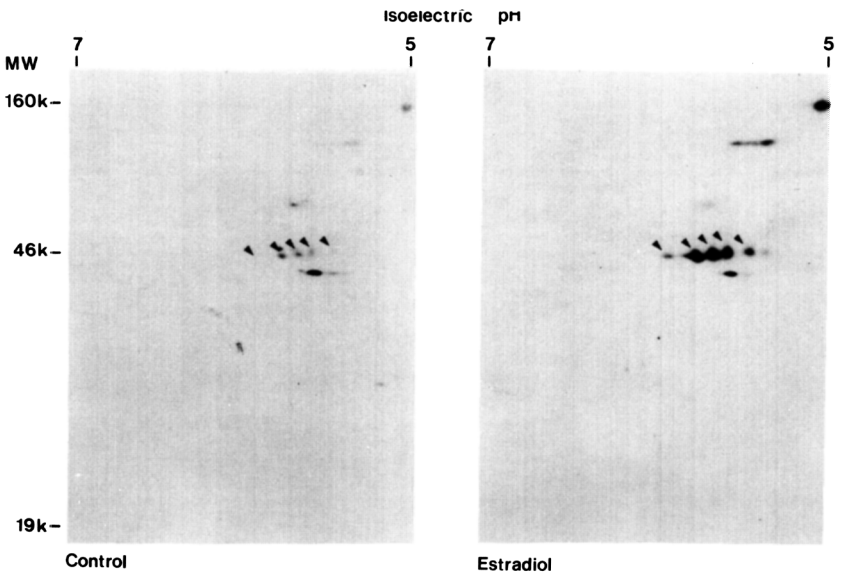

Figure 2 : Effect of estradiol on the synthesis of secreted proteins in MCF_7 cells : Fluorograms of secreted proteins labelled with (^{35}S)-methionine and analysed by two dimensional electrophoresis as described in the Methods. The cells were cultured for 2 days either in the absence (control) or presence (estradiol) of 0.1 nM estradiol. The arrows indicate the estradiol induced 46,000 k protein resolved into 5 components.

sed under our conditions with molecular weights between 15,000 and 160,000 daltons. Estradiol stimulated the synthesis of 5 of these proteins, however the 160,000 and 46,000 dalton proteins were the most dramatically stimulated. The effect shown in Fig. 1 with 0.1 nM estradiol was near maximal since very little additional stimulation was seen with any estradiol concentration up to 1 µM. Tamoxifen (1 µM) was found to be a pure estrogen antagonist in this system, since at concentrations up to 1 µM, it did not induce the estrogen induced proteins (fig. 1 track 3), while a 10^4 fold excess of tamoxifen totally prevented the induction by 0.1 nM estradiol (track 4).The induction of these proteins was specific for estrogens since 5 α -dihydrotestosterone, dexamethasone or progesterone were inactive at concentrations up to 0.1 µM. The 46,000 dalton protein, induced by estradiol, migrated as a broad band on SDS polyacrylamide gels and we therefore analysed the secreted proteins using the two dimensional gel technique described by O'Farrell (7). Figure 2 shows that only a few of the secreted proteins are seen on pH 5-7 isoelectric focussing gels but that most of these are stimulated by estrogen.

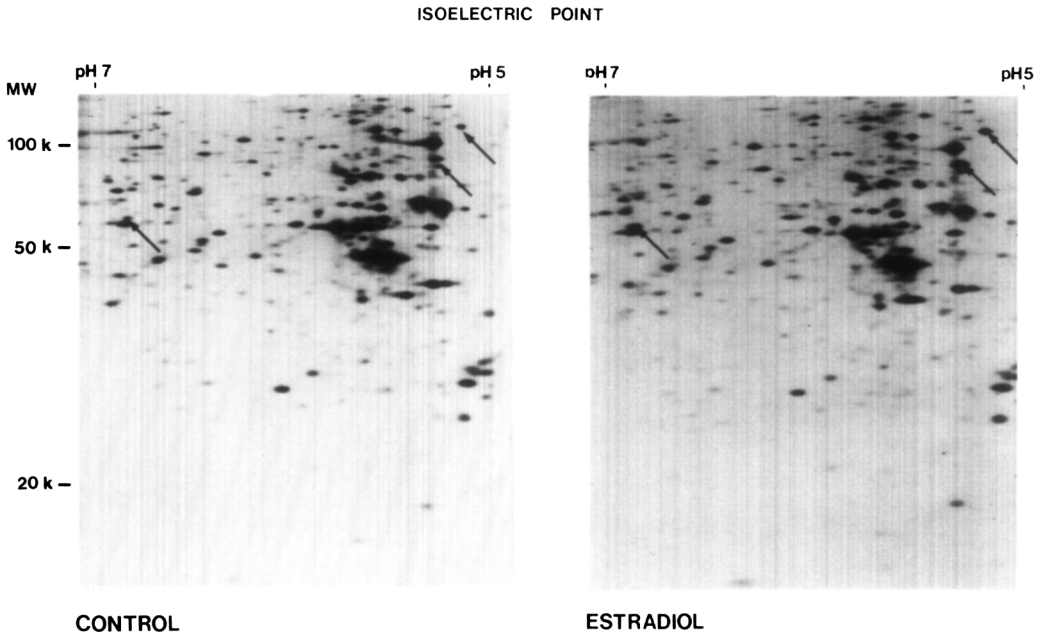

Figure 3 : Effect of estradiol on the synthesis of intracellular proteins in MCF_7 cells : Fluorograms of intracellular proteins, labelled with (^{35}S)-methionine and analysed by two dimensional electrophoresis. The cells were cultured for 2 days either in the absence (control) or presence (estradiol) of 0.1 µM estradiol. The arrows indicate the estradiol induced proteins.

They were either resolved as single spots or as a series of spots in which the more acidic components were always heavier. This latter pattern was seen for the 46,000 dalton protein induced by estradiol which was resolved into 3 major and 2 minor components. This pattern is typical of glycoproteins which vary only in the degree of sialyation (8) and accounts for their migration as a relatively broad band on one dimensional gels. We have also examined the intracellular proteins on two dimensional gels to see whether any of the proteins found in the medium were also found in the cell and whether we could detect any intracellular estrogen induced proteins. Fig. 3 shows the pattern obtained from cells cultured in the presence and absence of estradiol. About 250 proteins were visualised under these conditions of which 3 were found to be consistently stimulated by estradiol (3 experiments). However, these proteins were also present in the control cells and the degree of stimulation was much less dramatic than that seen for the 46,000 dalton secreted proteins. Only the most abundant intracellular proteins

could be detected on the two dimensional gels of the secreted proteins. For instance, the protein of molecular weight 40,000 daltons and of isoelectric pH 5.7, which migrates just below the 46,000 dalton estradiol induced secreted protein (Fig. 2) is a major intracellular protein but is only a minor protein in the medium. We therefore conclude that most of the proteins found in the medium are not due to contamination by intracellular proteins, even though the incorporation of (^{35}S)-methionine into secreted proteins only accounts for 0.3 % of the incorporation into intracellular proteins.

DISCUSSION

We have shown that estradiol induces several secreted and non-secreted proteins in MCF_7 cells. The effect on the secreted proteins is the most dramatic and is clearly visible on one dimensional polyacrylamide gels. Conversely, the effects of estradiol on the intracellular proteins are more subtle and limited since only 3 proteins out of 250 visualised on 2 dimensional gels are stimulated. The response is specific for estrogen as 5α-dihydrotestosterone, dexamethasone and progesterone do not induce the estrogen induced proteins. In addition, no other secreted proteins are induced in MCF_7 cells by these other hormones, even though these cells contain progesterone, androgen and glucocorticoid receptors. The results with tamoxifen are interesting because this antiestrogen is commonly used in the treatment of breast cancer and because it inhibits the growth of MCF_7 cells (2). We find that tamoxifen does not induce the estrogen induced proteins but totally inhibits their induction by estradiol. This system appears therefore useful for studying the mechanism of action of antiestrogens and for screening potential antiestrogens for use in human breast cancer.

The identity and function of the estrogen induced proteins are unknown. One of the induced intracellular proteins could be the progesterone receptor as this protein is increased by estrogen in MCF_7 cells (4). Among the estradiol induced proteins in mammals, the most extensively studied is that found in the uterus (9). The molecular weight and kinetics of induction (unpublished) of the estradiol induced proteins in MCF_7 cells however suggest that they are different to that of the uterus. The secreted induced proteins are probably not the milk proteins casein or

α-lactalbumin because they have different molecular weights. However, these induced proteins could be responsible for or only related to the effects of estrogen on mammary tumour cell growth. In favor of these hypotheses, we have found that some of these secreted proteins are also induced by estrogens in another subline of MCF_7 provided by Marvin Rich and in another estrogen responsive human breast cancer cell line (Westley et al, in preparation). In addition,both the induced proteins and the growth of MCF_7 cells were stimulated by estrogen and inhibited by antiestrogens (2). Consequently, these induced proteins might be better markers to predict estrogen responsiveness in human breast cancer, than the estrogen and progesterone receptors, as estrogen (5) and antiestrogen (4) can sometimes induce the progesterone receptor without stimulating growth.

To conclude, the most remarkable finding is that one of the major secreted proteins of 46,000 daltons molecular weight is controlled by estrogen which makes the MCF_7 cell line an excellent in vitro system to study the regulation of gene expression and cell proliferation by estrogen and antiestrogen in human breast cancer.

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