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SECRETION OF TRANSFERRIN BY HUMAN BREAST CANCER CELLS

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Transferrin (Tf), the major iron-binding protein in the plasma of vertebrate species is an essential growth factor for cells in serum-free media and appears to be involved in the regulation of growth and differentiation of human tissues. We report here that human breast cancer cells secrete a factor immunologically similar to Tf. The secretion of Tf by the hormone-responsive cell-line MCF-7 is stimulated by 17β-estradiol and reduced by the antiestrogen 4-hydroxy tamoxifen. These data suggest that Tf secreted by breast cancer cells may be an additional autocrine growth factor confering selective advantages to rapidly proliferating breast cancer cells and perhaps permit tumor cell growth in poorly vascularized areas.

Transferrin (Tf), the major iron-transporting protein in the plasma of vertebrate species, is a glycoprotein of molecular weight 80 000 daltons (1). Tf, which is mainly synthesized in liver parenchymal cells, is a growth factor required for all proliferating cells in the body (2). In vitro, it is an essential component of defined medium (3) and monoclonal antibodies to Tf receptors inhibit cell growth (4).

There is now evidence that under certain conditions some normal tissues synthesize Tf permitting specialized proliferation and differentiation. Indeed Tf or Tf-like substances are released from peripheral nerves and promote growth and development of chicken embryo myoplasts (5), T4 inducer T lymphocytes, a specific subset of T lymphocytes, synthesize Tf which appears to be involved in an autocrine pathway functionally linked to the interleukin-2/interleukin-2-receptor autocrine loop (6), Sertoli cells of the testes synthesize Tf to provide proliferating spermatocytes with iron (7).

Recently, it has been demonstrated that normal mammary epithelial cells were also able to synthesize Tf, the production being low in the virgin glands and greatly increased during pregnancy and lactation (8,9). Since few Tf syntheses have been reported in cancer tissues, to our knowledge only in T lymphoma cells (10) and lung cancer cells (11), it was of interest to examine Tf secretion by metastatic breast cancer cells MCF-7 under basal and stimulation conditions.

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MATERIAL AND METHODS

Cell culture and radiolabeling procedures

The metastatic breast cancer cells MCF-7 were grown and maintained at 37° in a humidified atmosphere of 5 % CO2 in DMEM/F12 medium supplemented with 5 % fetal calf-serum and 5 µg/ml insulin.

One day before the beginning of the experiments, MCF-7 cells were seeded in 10 cm Petri dishes (20 000/cm2) in phenol-red free DMEM/F12 supplemented with 5 % charcoal-stripped fetal calf-serum and 5 µg/ml insulin.

The medium was replaced 24 h post-plating by phenol-red free DMEM/F12 supplemented with insulin (5 µg/ml) transferrin (30 µg/ml) and human fibronectin (1 µg/ml) with or without the appropriate compounds i.e. estradiol ($E2.10^{-8}$ M) or 4-hydroxytamoxifen ($4OH\text{-}Tam.10^{-6}$ M). After 24 h the medium was removed, fresh medium was added, and cells were further stimulated for 48 h.

The synthesized proteins were studied on day 3 of stimulation. Cells were incubated in methionine and phenol-red free Eagle's minimal essential medium (MEM) containing 5 µg/ml insulin with or without E2 or 4OH-Tam and 11 mM glucose ; after 60 min the medium was discarded and [^{35}S] methionine (100 µCi/ml) in the same MEM medium was added to the dishes for 5 h.

Immunoprecipitation procedures

After incubation trypsin inhibitors (Sigma 20 µg/ml) were added to the media which were then passed through 0.22 µm millipore filters, lyophilized and dissolved in 0.5 ml lysis buffer (1 % triton X-100, 0.5 % sodium deoxycholate, 0.1 % sodium dodecylsulfate (SDS), 0.02 % sodium azide, 0,008 % sodium fluoride in phosphate buffer saline (PBS) pH 7.4).

After thorough washings in PBS, the cells were scraped from monolayers with a rubber-policeman and aliquots were saved for DNA assays and cell counts. The remaining cells were centrifuged and the resulting pellets were suspended in 0.5 ml lysis buffer, homogenised using a polytron then gently stirred for 30 min at 4°. Homogenates were afterwards centrifuged at 4° for 60 min at 50 000 g.

Aliquots of cell lysates or media in lysis buffer were incubated overnight under agitation at 4° with polyclonal Tf antibodies (Peil-Freez, Eurobio), protein A sepharose with or without large excess of cold Tf (1.5 mg/tube) and subsequently washed six times in lysis buffer. The immunoprecipitated proteins were dissociated from protein A sepharose by boiling for 4 min in 40 µl electrophoresis sample buffer (2 % SDS, 10 % glycerin, 5 % β-mercaptoethanol, 0.001% bromophenol-blue in 0.0625 M Tris-HCl pH 6.8) and centrifuged in Eppendorf microtube.

Aliquots of supernatant liquids (2 x 5 µl) were directly counted in a β-scintillation counter, the other part (about 30 µl corrected as a function of cell number) was subjected to 13 % SDS polyacrylamide gel electrophoresis according to Laemli (12). Gels were stained with Coomassie blue, dried and processed for autoradiography using hyperfilm β-max (Amersham France).

Other analytical methods

DNA was assayed as described by Le Pecq and Paoletti (13). Cell counts were performed using 2 M Coulter counter (Coultronics France).

RESULTS

In the media, figure 1 (T line) shows basal secretion of immunoreactive material giving a 80 Kd molecular weight band immunologically identical to Tf. This secretion is specific since large excesses of cold Tf abolish the 80 Kd band (NS line).

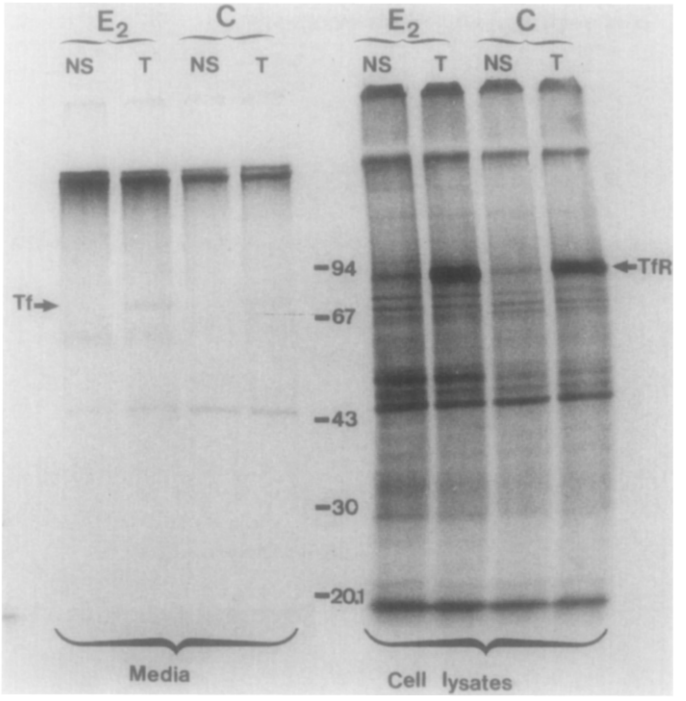

Figure 1 : Autoradiography of [^{35}S] methionine incorporated into proteins secreted by MCF-7 cells and immunoprecipitated with Tf-antibodies. Experiments were carried out after 3 days of stimulation with E2 . 10^{-8} M.
C : control = basal levels, T : total labeling, NS : labeling obtained in the presence of large excess Tf.

In the cell lysates specific 90 Kd proteins can be seen. These proteins correspond in all likelihood to the 180 Kd homodimer Tf-receptors since Tf is tightly bound to its receptor inside the cells (14). We and others have moreover described Tf receptors in breast cancer cells, and their regulation by E2 (15,16). Specific 80 Kd bands corresponding to Tf cannot be seen in cell lysates because of numerous non-specific bands concentrated in this area.

After 3 days of stimulation with E2.10^{-8} M, secretions increased both in media and cell lysates (fig 1) ; they were on the contrary reduced with 4OH-Tam.10^{-6} M (fig 2). These observations are quantified in table 1 where values represent Tf solely in the media and Tf plus Tf-receptors in cell lysates.

DISCUSSION

Many data have drawn a close relationship between growth factors and tumor cell growth. Tf appears to be centrally involved, at least as a co-factor, in the regulation of growth of both normal and malignant cells. Blocking interaction of Tf with its receptor results in growth inhibition of normal and transformed cells.

We have shown that MCF-7 cells which are metastatic cells of breast cancer, were able to synthesize Tf.

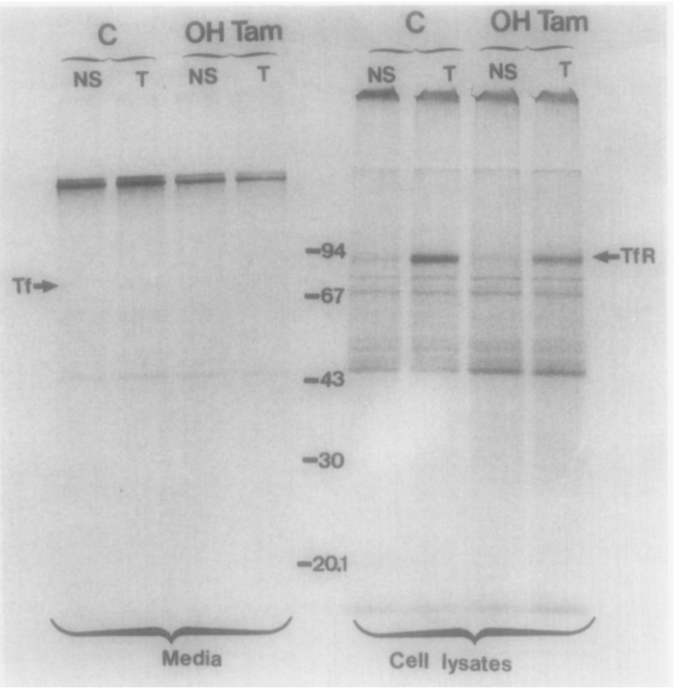

Figure 2 : Autoradiography of [^{35}S] methionine incorporated into proteins secreted by MCF-7 cells and immunoprecipitated with Tf-antibodies. Experiments were carried out after 3 days of stimulation with 4OH-Tam . 10^{-6} M.
C : control = basal levels, T : total labeling, NS : labeling obtained in the presence of large excess Tf.

Tf synthesis has also been reported in different normal or malignant tissues by some authors. Its regulation appears to be tissue-specific and in some cases physiological factors that modulate its secretion have been identified.

Table 1

QUANTITATIVE ANALYSIS OF SECRETED PROTEINS BY MCF-7 CELLS AFTER IMMUNOPRECIPITATION WITH Tf ANTIBODIES

	Media	Cell lysates
Control	100 %	100 %
E2 (10^{-8} M)	338 ± 128 %	226 ± 57 %
4OH-Tam (10^{-6} M)	57 ± 19 %	35 ± 8 %

Results (mean ± SD of 3 separate experiments) are expressed as percentage of control values.
The specific [^{35}S] methionine incorporations (total counts minus counts in presence of large excess Tf) for control values were 2.1 ± 0.9 and 7.2 ± 2.8 dpm/μg DNA in media and cell lysates respectively.

In the liver Tf synthesis is stimulated by iron-depletion and by steroid hormones (17). In the chicken, Tf is synthesized by the oviduct and its transcription is induced by estrogens (18), in rat Sertoli cells, Tf synthesis and secretion are regulated by testosterone and insulin (19). Finally Tf is regulated by pregnancy and extracellular matrix in the mouse mammary gland (18). We have shown that Tf secretion by MCF-7 cells is stimulated by E2 and reduced by the antiestrogen 4OH-Tam.

In other cancer cell lines such as NCI-H510 cells derived from small cell lung cancer, Tf synthesis increases more than 10-fold when cells enter active phase of the cell cycle and this increase is seen before large increases in Tf-receptor expression (11), the authors postulated that Tf synthesis by malignant cells was ultimately related to an iron requirement for cellular proliferation.

Tf, however, may be involved in other regulatory functions. Developmental changes in a number of tissues are often associated with increased rates of N-glycosylation and sometimes with the appearance of novel glycoproteins (21). Indeed Tf has been implicated in tissue differentiation of metanephric mesenchyme in organ culture (22), moreover Tf derived from suppressor T-cell has been reported to down-regulate the production of hematopoietin by T4 cells (23) and Tf derived from T-lymphoma cells to inhibit normal T-cell proliferation (10).

In normal rabbit mammary gland, the tissue's capacity to synthesize Tf increases from late pregnancy throughout lactation concomitantly with the synthesis of α-lactalbumin and casein (9). The discovery that Tf is actually synthesized by the mammary gland of rabbits (9) and mice (8) raises the possibility that Tf synthesis by mammary epithelium may be involved in the complex differentiation of mammary gland during pregnancy.

Our data suggest that Tf synthesized by MCF-7 cells may act as part of an important autocrine mechanism. The term "autocrine" has been used to describe self-stimulation processes of a cell producing a growth promoting factor and its specific membrane receptor (24). Syntheses of Tf and Tf-receptors by MCF-7 cells may confer selective growth advantages to these rapidly proliferating cells and perhaps permit tumor cell growth "in vivo" in areas not well vascularized. Additional Tf functions in breast tumor cells, unrelated to cell growth and iron requirement, have now to be considered.

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