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ESTROGEN AND INSULIN MODULATION OF INTRACELLULAR INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS IN HUMAN BREAST CANCER CELLS : POSSIBLE INVOLVEMENT IN LYSOSOMAL HYDROLASES OVERSECRETION

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SUMMARY : Differences in insulin-like growth factor binding proteins (IGFBPs) expressed within estrogen receptor positive (ER+, MCF-7/6) and negative (ER-, MDA-MB-231) human breast cancer cells cultured in chemically defined medium were observed. In the absence of insulin, 17 β-estradiol affects this expression in ER+ cells by significantly reducing 34 and 28 kDa species. In ER+ cells, insulin appears to minimize the estrogen induced reduction of these 34 and 28 kDa IGFBPs and stimulates a 24 kDa type. We suggest that through its association with a given IGFBP, insulin-like growth factor-II (IGF-II) directs lysosomal enzymes to secretion by its binding to the mannose-6-phosphate/IGF-II receptors present in the Golgi apparatus. Alternatively, the association of IGF-II with another IGFBP would inhibit this binding and lead to its autocrine or intracrine mitogenic action via the IGF-I receptor. © 1993 Academic Press, Inc.

Proteases, including lysosomal enzymes, are oversecreted by metastatic cells and seem to play a major role in tumor cell dissemination (1). This has been extensively documented for the lysosomal protease cathepsin D in human malignant breast cancer cells (2-4). Its oversecretion, as a 52 kDa autoactivable precursor, is regulated by estrogens in breast cancer cells expressing the estrogen receptor (ER+ cells) and is constitutive in cells lacking this receptor (ER- cells) (4). Secretion of soluble lysosomal enzymes by these cells appears to result, at least partially, from a defect in the targeting from their site of synthesis to their normal intracellular site. The shunt between the secretory and the lysosomal pathways occurs in the Golgi apparatus where most lysosomal enzymes are normally captured by the cation-independent mannose-6-phosphate/insulin-like growth factor-II (M6P/IGF-II) receptor and transferred to the endosomal/lysosomal compartment (5).

IGF-II and insulin-like growth factor binding proteins (IGFBPs) are produced and secreted by human breast cancer cells as well as by many other malignant cell types (6-8). IGF-II is a good mitogen for these cells and acts presumably via the IGF-I receptor while the IGFBPs seem to modulate the binding of IGFs to their receptors (9-11). IGF-II also binds with high affinity to a different site of the receptor recognized by phosphomannosylated lysosomal enzymes and interferes with their binding (12). IGF-II could therefore be the major regulator of the oversecretion of lysosomal enzymes by malignant cells, depending on the type or form of

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IGFBP to which it is bound intracellularly. Associated with an IGFBP which favors its binding to its receptor IGF-II would bind to it in the Golgi, thereby inhibiting or restricting the binding of lysosomal enzymes and deviating them into the secretory pathway. On the other hand, if IGF-II is associated with a form of IGFBP inhibiting its binding to the M6P/IGF-II receptor it will be driven into the secretory pathway allowing the phosphomannosylated enzymes to be normally targeted to lysosomes. IGFBPs would be under the control of estrogens in ER+ cells.

To test this "intracrine" hypothesis, we first analyzed the IGFBPs present within ER- human breast cancer cells and within ER+ cells exposed or not to estrogens. We report the different patterns of IGFBPs present in ER- and ER+ cells and their modulation by estrogens in ER+ cells. We also found an effect of insulin on IGFBPs present in ER+ cells. These results provide direct evidence in favor of our starting hypothesis and emphazise the possibility of an intracrine mode of action of IGF-II in the regulation of intracellular transport processes.

MATERIALS AND METHODS

Cell culture : The human breast cancer cell lines were obtained from Prof. M. Mareel (Gent, Belgium). ER+ cells (MCF-7/6) were cultured in DMEM/F12 medium without phenol red and putrescine (Gibco-BRL) supplemented with bovine serum albumin (BSA) (receptor grade, Serva) 200 µg/ml, transferrin (Sigma) 10 µg/ml and insulin (Novo) 1 µg/ml. For the last 24h of culture, the cells were deprived of insulin or not. ER- cells (MDA-MB-231) were cultured in DMEM medium supplemented with 10% fetal calf serum (Boehringer, Belgium) since they cannot be grown in the defined medium. The last 24h of culture were performed in the culture medium used for ER+ cells with or without insulin. To test the effects of estrogens on ER+ cells, 10 nM 17 β-estradiol (E_2) (Sigma) were added to the culture medium from the beginning of the culture.

Analysis of IGFBPs : Cells were harvested at about 90% confluence. They were first washed twice with phosphate buffered saline (PBS) at 4°C, then scraped with a rubber policeman, and finally washed again twice with cold PBS. The pellets were then resuspended in the extraction solution (Tris-HCl 100 mM, pH 7.4; 0.5% Triton X-100; EDTA 1mM; pepstatin 1 µM; aprotinin 0.3 µM; leupeptin 1 µM) at 4°C. After a 15 min. incubation on ice, the extracts were centrifuged at 15 000 g for 15 min., and the supernatants diluted 1/1 with the electrophoresis sample buffer (14) containing no β-mercaptoethanol. Samples were boiled for 3 minutes and submitted to SDS-PAGE (33 µg protein per lane as determined by the Folin-Ciocalteu method with bovine serum albumin standards) according to the method of Laemmli (13). Gels were then blotted onto nitrocellulose (Amersham) and membranes were ligand-blotted using [^{125}I]IGF-II (Amersham) as described elsewhere (14). Autoradiography was performed on Amersham hyperfilm with one intensifying screen. Specificity of detection was controlled as follows. Extracts of MCF-7/6 and MDA-MB-231 cells were electrophoresed (2 lanes each) and transferred to a nitrocellulose sheet. One half of the sheet was incubated with [^{125}I]IGF-II and the other half with the same dose of [^{125}I]IGF-II supplemented with 200 nM unlabeled IGF-II (gift of Eli Lilly Co, Indianapolis). Unlabeled IGF-II totally inhibited the signals equivalent to those obtained on the first half of the sheet. Autoradiograms were analyzed using the NIH Image 1.47 software. The relative amount of each major band was assessed on the basis of its surface area and mean density.

RESULTS AND DISCUSSION

In order to explore the possibility that, under the influence of estrogens, IGFBPs could intracellularly affect the interaction of IGF-II and phosphomannosylated lysosomal enzymes with the M6P/IGF-II receptor, we examined the IGFBP types present in ER- and ER+ human

breast cancer cells in the presence or absence of estrogens. It has already been established (15-17) that there are differences in the patterns of IGFBPs secreted by ER- and ER+ breast cancer cells, and that estrogens alter the pattern of IGFBPs detected in the culture medium of ER+ cells (18-20).

Figure 1(a) shows the comparison of MCF-7/6 and MDA-MB-231 cells that were deprived of insulin and the effects of 10 nM E_2 on MCF-7/6 cells. This first confirms that the IGFPBs patterns of MCF-7/6 and MDA-MB-231 cells are different. In the MCF-7/6 extract, three main bands are detected : one major band with an apparent molecular weight of 34 kDa (presumably IGFBP-2) and two minor ones of 28 and 24 kDa (presumably IGFBP-5 and IGFBP-4, respectively). Recently, it has been shown by northern blotting that mRNAs corresponding to these IGFBPs are expressed by MCF-7 cells (18-20). In MDA-MB-231 cells there is only one clearly detectable band with an apparent molecular weight of 24 kDa. The total amount of IGFBPs in the ER- cells also appears to be quite lower. Furthermore, there is a marked effect of E_2 on the expression of these proteins. In the presence of estrogens, MCF-7/6 cells express three similar IGFBPs but with an important decrease in the 28 kDa form. The 34 kDa IGFBP is decreased to a lesser extent while the 24 kDa band is practically unaffected. These results confirm the existence of a specific expression of some IGFBPs in ER+ and ER- breast cancer cells. They are also in good agreement with the observation that estrogens may be able to regulate the expression of IGFBPs mRNAs (18-20). Figure 1(b) shows that, as expected, E_2 has no effect on IGFBPs expression in ER- MDA-MB-231 cells. This indicates the involvement of the estrogen receptor in this regulation process.

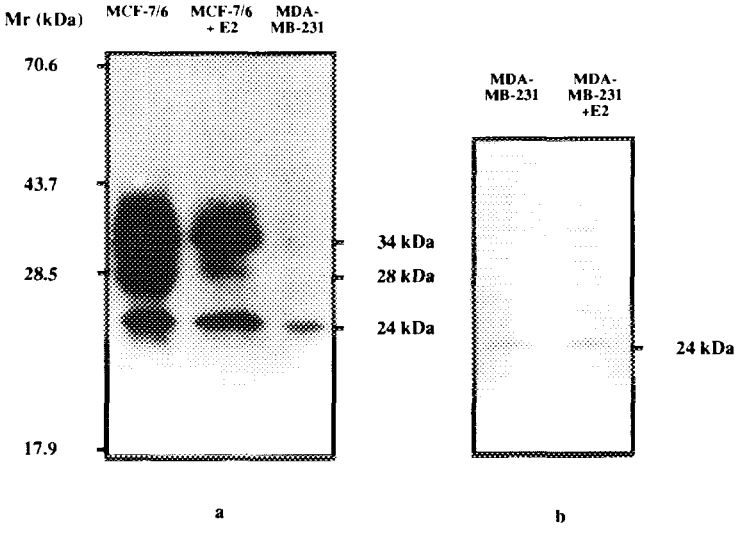

Figure 1. (a) Ligand blot analysis of ER+ (MCF-7/6) and ER- (MDA-MB-231) human breast cancer cells deprived of insulin for the last 24h of culture. Effect of 10 nM 17 β-estradiol on the expression of IGFBPs in MCF-7/6 cells. Thirty three µg protein/lane was loaded onto SDS-polyacrylamide gel electrophoresis under non-reducing conditions. The proteins were transferred to nitrocellulose and IGFBPs detected by incubation with [^{125}I]IGF-II and autoradiography; (b) control for the absence of effect of E_2 on ER- MDA-MB-231 cells [conditions similar to figure 1(a)].

Figure 2 shows that E_2 also exerts an effect when ER+ cells are continuously cultured in the presence of insulin. Insulin itself seems to influence the production of IGFBPs by the ER+ cells whereas it has no effect on ER- cells (data not shown). This insulin effect on ER+ cells is not surprising since insulin has already been shown to down-regulate the expression of IGFBP-2 mRNA in rat hepatocytes (21) and of IGFBP-1 mRNA in hepatoma cells (22, 23), as well as affecting the IGFBPs levels *in vivo* (24). In our experiments, insulin deprivation increases the amount of the 24 kDa IGFBP (1.8-fold increase) and it could thus also be a negative regulator of IGFBPs in MCF-7/6 breast cancer cells. Without insulin, E_2 lowers the amount of the 28 and 34 kDa IGFBPs (61.5 and 4 fold decrease respectively) whereas in the presence of insulin this down-regulation is less pronounced (1.6 and 1.1 fold decrease) and accompanied by an up-regulation (2.8 fold increase) of the 24 kDa IGFBP (figure 2). The modes of action of E_2 and insulin in the regulation of IGFBPs remain to be investigated. Insulin could act via its own receptor or via the IGF-I receptor as IGF-I is also a potent regulator of IGFBPs in human breast cancer cells (18). It should also be noticed that the extent of insulin effect varies slightly from one experiment to the other.

These results show very clearly that studies concerning IGFBPs and IGFs using cells in culture should be performed with care. Indeed a great variety of extracellular factors may influence the intracellular levels and types of IGFBPs and the data will be very difficult to interpret unless well defined culture media and conditions are used.

A marked estrogen-induced decrease in the intracellular pool of the 28 and 34 kDa IGFBPs is observed in MCF-7/6 cells. When these cells are not deprived of insulin, the effect of

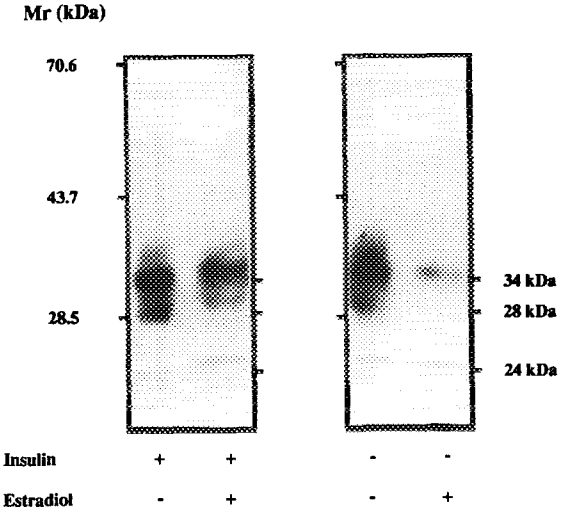

Figure 2. Ligand blot analysis of MCF-7/6 cell extracts. Effects of 10 nM 17 β-estradiol and 1μg/ml insulin on the expression of intracellular IGFBPs. Thirty three μg protein/lane was loaded onto SDS-polyacrylamide gel electrophoresis under non-reducing conditions. The proteins were transferred to nitrocellulose and IGFBPs detected by incubation with [^{125}I]IGF-II and autoradiography. The exposition time of the film is shorter than that used in figure 1.

estrogens is less important. We have as well confirmed recent results showing that estrogens induce an increase in the amounts of these 24, 28 and 34 kDa IGFBPs contained in MCF-7 conditioned media (18, 19). The most important effect is on the 24 kDa form. The modifications affecting the intracellular IGFBPs (detected by western ligand blotting as opposed to northern blotting experiments) could result from an effect on the rate of synthesis of these proteins, on their secretion or on both. An effect on the secretion should always be accompanied by an effect on the culture medium composition. This is not necessarily the case for an effect on the rate of synthesis.

In the absence of insulin, the amount of the 24 kDa IGFBP present within the cells is almost not affected under estrogen stimulation. In conditioned media, an important increase in the abundance of this form has been described, suggesting that estrogens could stimulate both synthesis and secretion of this IGFBP. Estrogens also induce an important decrease of the 28 and 34 kDa IGFBPs within the cells and these IGFBPs appear more abundant in the conditioned media. So in this case, estrogens could affect secretion to a greater extent as compared to synthesis. This latter action cannot be excluded since estrogens up-regulate the mRNAs corresponding to these IGFBPs (18, 19), but one should notice that mRNA levels do not absolutely correlate with the corresponding protein levels.

The estrogen and insulin induced modulation of intracellular IGFBPs described here in cultured breast cancer cells draws the attention to a probable intracellular regulatory role of IGFBPs. An extracellular role was already advocated in the growth-stimulatory effects of estradiol (18).

We suggest that the IGFBPs also function in the intracellular control of the oversecretion of lysosomal enzymes and consequently of the metastatic process. It has previously been postulated that the oversecretion of lysosomal enzymes by breast cancer cells results from a saturation mechanism affecting the M6P/IGF-II receptor, deviating these enzymes into the secretory pathway (25). This saturation process was supposed to result from a negative regulation of the receptor gene by estrogens in ER+ cells and from an increase in the synthesis of enzymes. This type of mechanism involving the M6P/IGF-II receptor seems very plausible since E_2 also induces the secretion of other phosphomannosylated lysosomal hydrolases such as β-hexosaminidase and β-galactosidase but does not influence acid phosphatase which is not routed to the lysosomes by this receptor (26). Furthermore, cathepsin D secreted by hepatoma cells contains twice as many mannose-6-phosphate residues as cathepsin D from normal hepatocytes, whereas the core protein is identical in the two cell types (27).

We therefore propose to extend this hypothesis by suggesting : (a) that the interaction of IGF-II with its receptor (which it necessarily meets in the Golgi) plays a major role in the saturation process; (b) that IGFBPs can, under the influence of estrogens in ER+ cells or constitutively in ER- cells, regulate the saturation of the M6P/IGF-II receptor by IGF-II; (c) that IGF-II can either saturate the M6P/IGF-II receptor or function intracellularly or extracellularly as a mitogen by interacting with the IGF-I receptor (9-11), depending on the type or form of IGFBP it interacts with intracellularly. It has been shown (28) that, in NIH-3T3 cells, the overexpression of IGF-II did not affect the sorting of newly synthezised phosphomannosylated

proteins. This is interesting as in this type of experiment, if IGF-II is overexpressed in the Golgi, IGFBPs are not affected and the excess of IGF-II might also well be associated with an IGFBP inhibiting its action on the M6P/IGF-II receptor. So, finally, these results might well be those expected when considering our hypothesis. Within the Golgi network, the important factor is probably not simply the presence of one IGFBP type and/or the absence of another. What might actually be more important is the ratio of one IGFBP to the others. As a matter of fact, one can suppose that the type of IGF-IGFBP complex dominating intracellularly is the result of a competition between the different IGFBPs present in the cell. For example, in our case the ratio of the 24 kDa IGFBP to the 28 kDa one varies in response to estrogens. This ratio is inverted, rising from 0.07 to 1.8 in the case of insulin deprivation. Moreover, our hypothesis confers a role to the association between the M6P/IGF-II receptor and IGF-II, since any clear function has never been attributed to it so far (29). The concept of an intracellular interaction of a polypeptidic growth factor with its receptor is not new and has been evoked in the case of platelet-derived growth factor receptor and *sis* oncogene products (30-32). Evidence has also been presented in its favor in the case of transforming growth factor-α which seems to stimulate cell growth without the requirement of an externalization after synthesis (33).

According to this hypothesis, IGF-II would be able, through its intracellular association with different IGFBPs, either to redirect lysosomal enzymes towards the extracellular medium or to act as a mitogen. Possessing an affinity for both the M6P/IGF-II receptor and the IGF-I receptor, IGF-II could therefore alternatively govern the two major characteristics of cancer cells, namely tissular invasion and proliferation.

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