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Progesterone-induced secretion of growth hormone, insulin-like growth factor I and prolactin by human breast cancer explants

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ABSTRACT

The aim of the study was to evaluate the potential of human breast cancer tissue to secrete growth hormone (GH), insulin-like growth factor I (IGF-I) and prolactin in response to 10^{-7} M progesterone stimulation. Explants were divided according to estrogen receptor (ER)/progesterone receptor (PR) phenotype (ER(-)PR(-); ER(+)PR(-); ER(+)PR(+); ER(-)PR(+)). Our results show distinct differences in cultured breast cancer tissue responses to progesterone stimulation with regard to secretion of proliferative agents such as GH, IGF-I and prolactin. All but ER(-)PR(-) breast cancer cell types responded *in vitro* to progesterone stimulation with an increase in local GH secretion, while in non-malignant tissue progesterone induced local GH secretion only in PR(+) cells. Moreover, only in PR(+) cells did progesterone stimulate local IGF-I and prolactin secretion, in both malignant and non-malignant tissue. This study provides evidence for the first time that in PR(+) breast cancer tissue, progesterone may increase GH, prolactin and IGF-I secretion in both malignant and surrounding non-malignant tissue. These hormones

may act as local growth factors that stimulate the proliferation of mammary tumors.

INTRODUCTION

There is still no precise account of the role of progestins in human breast cancer development. Depending on the dose and the experimental model, progesterone has been reported to either stimulate or inhibit the growth of experimental mammary tumors¹⁻⁴. The highest proliferation rate was observed in women receiving progestin-only formulations of contraceptives, suggesting a strong mitogenic action of progestins on mammary epithelium^{3,5}. Soderqvist⁵ showed that both endogenous progesterone and exogenous progestins augment proliferation of breast epithelial tissue. On the other hand, Botella *et al.*⁶ showed that the progestins nomegestrol acetate, ORG2058, norethindrone acetate, medroxyprogesterone

acetate and promegestone (R5020) induce a dose-dependent inhibition of cell proliferation as measured by [³H]thymidine incorporation. Horwitz *et al.*⁷ showed that progesterone added to the medium of T47Dco human breast cancer cells is metabolized with a half life of 2–4 hours, and Wiebe *et al.*⁸ later suggested that a change in *in situ* progesterone metabolism, resulting in an increased 5 α -pregnane-3,20-dione : 3 α -hydroxy-4-pregnen-20-one ratio, may induce breast cancer by promoting increased cell proliferation, whereas increases in 4-pregnenes may retard these tumorigenic processes; these studies may help explain the discrepancies found in different laboratories.

A retrospective cohort study, carried out by Schairer *et al.*⁹, showed that long-lasting (over 5 years) combined estrogen–progestin hormone replacement therapy (HRT) is associated with an increased risk of breast cancer compared with estrogen-only HRT. However, Ross *et al.*¹⁰ found that continuous estrogen–progestin HRT carries a smaller risk of breast cancer than sequential HRT.

The other interesting and unresolved question is the dependency of progesterone's action on sex steroid-receptor phenotypes. Recently, there has been an enormous amount of information on the oxidative pathway particularly in tumors of estrogen receptor-positive (ER(+)) phenotype. Castagnetta *et al.*¹¹ showed a prevalence of reductive metabolism (estrone to estradiol) in ER(+) cells, while oxidative pathways (estradiol to estrone) were largely dominant in mammary cells that expressed ER poorly. The other possibility is the mitogenic action of progesterone via growth hormone (GH), prolactin and insulin-like growth factor I (IGF-I) secretion, hormones that have growth-promoting characteristics. In animal models, progestin administration results in local mammary production of GH in both normal and malignant breast tissue. The highest level of GH mRNA has been found after the prolonged exposure of dogs and cats to progestins¹². Prolonged progestin administration has previously been shown to induce circulating GH excess^{13–16}. Selman *et al.*¹⁷ showed that progestin-induced GH excess originates from foci of hyperplastic ductular epithelium of the mammary gland in dogs. Surprisingly, there are no data reporting the expression of the GH gene in the human mammary gland, and the question of whether GH expression or local

mammary production of GH is induced by progesterone needs to be resolved.

Treatment of the T47D human breast carcinoma cell line with a synthetic progestin has been shown to induce a time- and dose-dependent increase in insulin receptor content as well as in IGF-II concentration¹⁸. Insulin alone has a small effect on cell growth. Exposure to progesterone markedly potentiates the effect of insulin. Papa *et al.* suggested the importance of progestin–insulin interaction for breast cancer growth regulation¹⁹.

Tumors are routinely classified as ER(+)/PR(+) when the tissue expresses both estrogen and progesterone receptors; as ER(-)/PR(-) if neither type of receptor is expressed; as ER(+)/PR(-) when more than 50% of tissue expresses estrogen receptors; and finally as ER(-)/PR(+) if expression of progesterone receptors is present in more than 50% of cells.

The possible mechanisms by which progestins may affect mammary gland proliferation need further investigation. The aim of the study was to evaluate the ability of human breast cancer tissue to secrete GH, IGF-I and prolactin in response to progesterone stimulation, and whether the response is dependent on sex steroid-receptor phenotype.

MATERIAL AND METHODS

Both breast cancer tissue and non-malignant mammary tissue were obtained during radical mastectomies performed at the Gynecological Oncology Department of the Jagiellonian University in Krakow, Poland. Explants of malignant and surrounding non-malignant tissue were prepared under the close supervision of a pathologist, to prevent interfering with further pathologic evaluation of malignant lesions of the breast. Some of the explants were fixed in 10% buffered formalin for 48 hours at room temperature, dehydrated and embedded in paraffin using a standard procedure. Sections 5 μ m thick were mounted on slides and deparaffinated before processing for receptor determination by immunohistochemistry. Type and prevalence of steroid tissue receptors were evaluated using commercial monoclonal antibody kits manufactured by DACO A/S (Denmark). Each section was assessed to demonstrate the presence of estrogen receptor (ER) and/or progesterone receptor (PR). Tumors were classified

as ER(+)/PR(+), ER(-)/PR(-), ER(+)/PR(-) or ER(-)/PR(+).

Mammary gland *in vitro* incubation

The remaining explants from the malignant tissue and surrounding non-malignant tissue were trimmed into 0.5 cm³ pieces, weighed and incubated in 2 ml of M199 medium containing 5% calf serum. Results obtained by means of immunohistochemistry were later matched with those from culture. Four explants obtained from malignant or non-malignant tissue of each individual patient were placed in culture tubes and incubated for 48 h in control medium or with the addition of 10⁻⁷ M progesterone. This resulted in 16, 48, 16 and 12 malignant and non-malignant ER(-)/PR(-); ER(+)/PR(+); ER(-)/PR(+); and ER(+)/PR(-) tissue explants, respectively. Experimental tubes with explants were shaken gently at 37°C. The media were continuously gassed with 5% CO₂/95% air during incubation. After 48 hours' incubation, the collected media were frozen at -20°C for evaluation of human GH, IGF-I and prolactin levels in the media.

Hormone concentration evaluation

All culture media were stored at -20°C until assayed. IGF-I levels were measured using a Biosource Europe S.A. RIA kit (Belgium). Sensitivity was 0.25 ng/ml; coefficients of variation within and between assays were 4.1% and 9.3%, respectively; cross-reactivity with insulin was 0.001%, with human GH 0.01%, and with IGF-II 0.2%. Human GH levels were estimated with an OP (Swierk, Poland), RIA kit. Sensitivity was 0.5 ng/ml; and coefficients of variation within and between assays were 2.1% and 2.7%, respectively. For prolactin assessment, a Spectra (Orion Diagnostica, Finland) RIA kit was used. Sensitivity was 0.6 ng/ml, the coefficients of variation within and between assays were 2.3% and 3.4%, respectively; cross-reactivity with human GH was 0.039%; and there was no cross-reactivity with follicle-stimulating hormone, luteinizing hormone, thyroid-stimulating hormone, human chorionic gonadotropin or human placental lactogen (PL).

STATISTICAL ANALYSIS

All data points are expressed as mean ± SEM. Significant differences between the concentrations of GH, prolactin and IGF-I in malignant and non-malignant tissue and between control and progesterone-treated explants were compared by analysis of variance (ANOVA) and using Duncan's new multiple-range test. The normal distribution of data was checked by the Mann-Whitney U test.

RESULTS

In highly differentiated malignant tissue ((ER(+)/PR(+)) there was no difference between basal GH secretion by malignant and non-malignant tissue (0.03 ± 0.001 ng/10 mg tissue vs. 0.02 ± 0.002 ng/10 mg tissue, respectively). However, progesterone produced a decrease in GH secretion in non-malignant tissue (0.05 ± 0.001 ng/10 mg progesterone-stimulated tissue vs. 0.03 ng ± 0.001 ng/10 mg control tissue; *p* < 0.05) (Figure 1a).

Basal IGF-I secretion in ER(+)/PR(+) malignant tissue was twice as high as that of non-malignant tissue (5.7 ± 0.5 ng/10 mg control malignant tissue vs. 2.9 ± 0.2 ng/10 mg control non-malignant tissue *p* < 0.01). Progesterone increased IGF-I secretion in both malignant and non-malignant tissue (7.8 ± 0.6 ng/10 mg progesterone-stimulated malignant tissue vs. 5.7 ± 0.5 ng/10 mg control malignant tissue; 3.8 ± 0.3 ng/10 mg progesterone-stimulated non-malignant tissue vs. 2.9 ± 0.2 ng/10 mg control non-malignant tissue, *p* < 0.05) (Figure 1b). Prolactin secretion from control and progesterone-stimulated tissue was higher in malignant ER(+)/PR(+) tissue than in non-malignant tissue (3.8 ± 0.2 ng/10 mg control malignant tissue vs. 2.1 ± 0.02 ng/10 mg control non-malignant tissue (55%); and 4.5 ± 0.1 ng/10 mg progesterone-stimulated malignant tissue vs. 3.2 ± 0.08 ng/10 mg progesterone-stimulated non-malignant tissue (71%). In contrast to GH and IGF-I, exposure to progesterone caused the highest prolactin rise only in ER(-)/PR(+) breast cancer tissue (*p* < 0.01) (Figure 1c).

Malignant ER(+)/PR(-) tissue GH secretion increased in response to progesterone stimulation (0.1 ± 0.02 ng/10 mg progesterone-stimulated tissue vs. 0.06 ± 0.001 ng/10 mg control tissue;

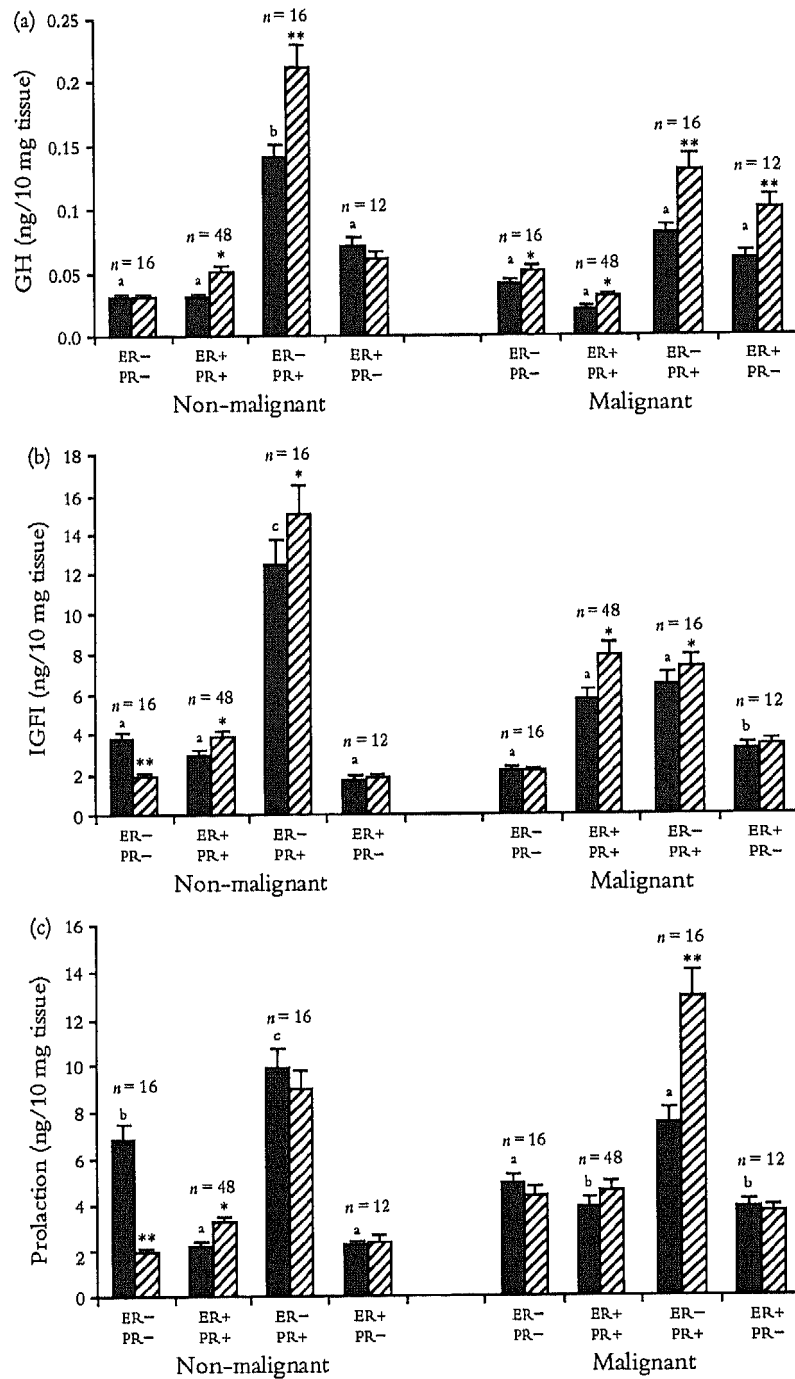


Figure 1 (a) Growth hormone (GH), (b) insulin-like growth factor I (IGF-I) and (c) prolactin release by explants of non-malignant and malignant tissue from non-hormone dependent (ER-PR-), estradiol receptor-positive and progesterone receptor-positive (ER+PR+), progesterone receptor-positive early (ER-PR+) and estradiol receptor-positive only (ER+PR-) breast cancer. Explants were cultured in M199 medium supplemented with 5% serum as a control medium (■) or with the addition of 10⁻⁵ M progesterone (▨). **p* < 0.05; ***p* < 0.01 for control vs. progesterone-stimulated explants; and a-a, no significant difference; a-b, *p* < 0.05; a-c, *p* < 0.01 for non-malignant vs. malignant tissue

$p < 0.01$) while in non-malignant tissue, progesterone exerted no influence on GH secretion (Figure 1a). Neither in malignant nor in non-malignant ER(+)/PR(-) tissue did progesterone influence IGF-I release (Figure 1b). The same pattern applied for prolactin secretion in malignant ER(+)/PR(-) tissue (Figure 1c).

Non-malignant ER(-)/PR(+) tissue under both basal and progesterone-stimulated conditions secreted a higher amount of GH than malignant tissue (Figure 1a). However, in both malignant and non-malignant ER(-)/PR(+) tissue, progesterone significantly increased GH secretion (0.21 ± 0.01 ng/10 mg progesterone-stimulated non-malignant tissue vs. 0.14 ± 0.04 ng/mg control non-malignant tissue; and 0.13 ng \pm 0.03 ng/10 mg progesterone-stimulated malignant tissue vs. 0.08 ng \pm 0.001 ng/10 mg control malignant tissue, $p < 0.01$) (Figure 1a). In this type of the tumor, IGF-I release followed the same pattern as GH. Control and progesterone-stimulated IGF-I secretion in malignant tissue was lower than in non-malignant tissue. However, progesterone exposure significantly ($p < 0.05$) augmented IGF-I secretion in both malignant and non-malignant ER(-)/PR(+) tissue (14.9 ± 0.5 ng/10 mg progesterone-stimulated non-malignant tissue vs. 12.4 ± 0.4 ng/mg control non-malignant tissue; and 7.3 ± 0.5 ng/10 mg progesterone-stimulated malignant tissue vs. 6.4 ng \pm 0.4 ng/10 mg control malignant tissue) (Figure 1b). Control prolactin secretion was lower in malignant ER(-)/PR(+) tissue (7.4 ± 0.04 ng/10 mg malignant tissue vs. 9.8 ± 0.08 ng/10 mg non-malignant tissue, $p < 0.05$), while a progesterone-stimulated prolactin secretion increase was found only in malignant tissue (12.8 ± 0.5 ng/10 mg progesterone-stimulated malignant tissue vs. 7.4 ± 0.01 ng/10 mg control malignant tissue, $p < 0.01$) (Figure 1c).

While progesterone did not change GH secretion in ER(-)/PR(-) non-malignant tissue, an increase in GH release was observed in malignant tissue (0.05 ± 0.001 ng/10 mg tissue in progesterone-stimulated malignant tissue vs. 0.03 ± 0.001 ng/10 mg in control malignant tissue, $p < 0.05$) (Figure 1a). Progesterone significantly decreased IGF-I secretion by non-malignant ER(-)/PR(-) tissue (1.9 ± 0.3 ng in progesterone-stimulated non-malignant tissue vs. 3.7 ± 0.4 ng/10 mg in control non-malignant tissue, $p < 0.01$),

with no such effect in malignant ER(-)/PR(-) explants (Figure 1b). Similarly, progesterone caused a decrease in prolactin secretion by non-malignant ER(-)/PR(-) tissue (1.9 ± 0.2 ng/10 mg progesterone-stimulated tissue vs. 6.8 ± 0.3 ng/10 mg control tissue), but had no effect on malignant ER(-)/PR(-) tissue (Figure 1c).

DISCUSSION

Progesterone and progestins have been shown to stimulate GH gene expression in both malignant and normal mammary glands of a bitch²⁰. These agents also increase the local secretion of IGF-I, IGF-II and insulin-like growth factor binding protein-3 (IGFBP-3) in dogs, cats and humans²¹. The maximal level of GH mRNA was found after prolonged progestin administration, which occurs in women taking monophasic oral contraception or continuous combined HR T²². Malignant breast tissue preserves GH and IGF-I local secretion, although this is less progesterone dependent¹³. Epithelial proliferation can be stimulated directly through GH binding to epithelial receptors or indirectly through stromal augmentation of IGF-I secretion²³. Prolactin as well as GH may be locally produced by both normal and neoplastic mammary glands. In humans, GH activates both prolactin and GH receptors, which leads to elevations in IGF-I as well as functionally active prolactin levels. IGF-I hyperactivity seems to be insufficient alone for activation of breast tumor formation, but together with prolactin, is able to induce pathological proliferation²⁴. Moreover, prolactin suppresses IGFBP-5, which is a potent inhibitor of IGF-I action²⁵. We have documented the response of breast cancer tissue to progesterone stimulation, demonstrating a significant increase in GH release regardless of receptor phenotype, which in unstimulated tissue occurred only in progesterone receptor-positive tissue. Progesterone induced a significant increase in IGF-I release in progesterone receptor-expressing malignant tissue, whereas basal IGF-I secretion was higher in malignant estrogen receptor-expressing tissue. Prolactin secretion augmentation in response to progesterone exposure was found only in PR(+)/ER(-) phenotype breast cancer tissue.

Growth hormone and IGF-I synergize with estrogen to induce normal mammary gland development. Estrogen receptor expression in

mammary gland epithelium is increased in animals after GH administration²⁶. A positive correlation has been found between IGF type I receptor (IGFR-I) and sex steroids receptors in mammary gland tumors²⁷. IGFR-I blockade inhibits the growth of breast cancer cell lines²⁸. Progesterone receptor signalling may have important consequences in terms of mammary development, particularly with regard to ductal versus alveolar growth²⁹. According to Nizze *et al.*³⁰, ER(+)/PR(+) and ER(-)/PR(+) phenotypes might be regarded as low risk, whereas ER(+)/PR(-) and ER(-)/PR(-) phenotypes may be high risk³⁰. This may partly agree with our findings of increased basal IGF-I secretion in estrogen receptor-expressing malignant tissue and a higher basal GH release in ER(-)/PR(-) breast cancer tissue. However one of the receptor phenotypes regarded by Nizze *et al.*³⁰ as low risk, i.e. ER(-)/PR(+), vigorously responded to progesterone stimulation in our study, with a rise in GH, IGF-I and prolactin secretion in malignant tissue and GH and IGF-I secretion in non-malignant tissue. These increases may create a permissive environment for epithelial proliferation.

The importance of locally produced prolactin in pathological conditions such as mammary tumor growth in women has been indicated in several studies^{31,32}. Prolactin receptors have been found in 40–70% of human breast tumors^{33,34}. There is no clear correlation between prolactinomas and mammary tumors in women. Ormandy *et al.*³⁵ showed that sex steroids and prolactin interact synergistically to control the neoplastic growth of the mammary gland. Prolactin receptor expression was linearly correlated with estrogen and progesterone receptor expression. Ormandy *et al.*³⁵ also suggested co-expression and cross-regulation of prolactin and sex steroid receptors providing a potential mechanism for the observed synergy between estrogen, progesterone and prolactin in the control of tumor growth. Progesterone

pre-treatment enhances the ability of prolactin to stimulate transcription in mammary gland malignant tissue³⁵. In our study, we found the highest basal and progesterone-stimulated prolactin secretion in both malignant and control tissue only if these tissues possessed progesterone receptors only, i.e. ER(-)/PR(+) tissue. It is also worth pointing out that there was a lack of progesterone-induced inhibition of prolactin and IGF-I release in sex steroid receptor-negative malignant tissue in our study.

Our results indicate that there are distinct differences in cultured breast cancer tissue responses to progesterone (10^{-7} M) stimulation with regard to secretion of proliferation-inducing agents such as GH, IGF-I and prolactin. All but ER(-)/PR(-) phenotype breast cancer explants of malignant tissue responded *in vitro* to progesterone stimulation with an increase in local GH secretion, while in non-malignant tissue progesterone increased local GH secretion only in PR(+) phenotype breast cancer. The increase in local IGF-I and prolactin secretion by both malignant and non-malignant tissue under the influence of progesterone *in vitro* was noted only in PR(+) phenotype breast cancer. These data for the first time point to a stimulatory action of progesterone not on proliferation of breast epithelial cells but on GH, prolactin and IGF-I secretion. Prolactin and GH are both mitogenic hormones. We propose that increased levels of prolactin, IGF-I and GH under the influence of progesterone in PR(+) phenotype breast cancer may act as local growth factors that stimulate the proliferation of mammary tumors.

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