Prolactin involvement in breast cancer

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Abstract

Normal development and differentiation of the mammary gland are profoundly influenced by prolactin (PRL). In rodent mammary cancer PRL plays a well defined role, but its role in human breast cancer has not been appreciated until recently. It is now clear that breast tissue, both normal and malignant, is a significant source of extrapituitary PRL. Thus an autocrine/paracrine role of PRL in human breast cancer may be invoked. Both PRL and PRL receptor mRNA are expressed in the vast majority of breast cancer biopsies independent of estrogen and progesterone receptor status. An autocrine/paracrine PRL acting in human breast cancer requires that this hormone's action be blocked at the cellular level, as opposed to suppressing the synthesis and secretion of pituitary PRL. Mutants of PRL or human growth hormone are being explored which act as selective PRL antagonists. In addition, tamoxifen has been shown to act locally at the target tissue by binding directly to the PRL receptor and thus inhibiting PRL's action. These strategies may have clinical relevance in treating PRL-responsive human breast cancer.

Breast cancer models and prolactin

Historically, the role of the peptide hormone prolactin (PRL) in human breast cancer has been an obscure one. It has been, and still is, difficult to assign a role of this hormone in either the etiology or progression of the disease. However, it is well established that PRL is intimately involved in development and differentiation of the normal gland in mammalian species, and in rodent model systems it is a key player in the development of mammary cancer. Much of the literature on human breast cancer and PRL appears to be contradictory. Thus, it has been difficult to establish a defined involvement of PRL in human breast disease. However, it is hard to believe that a hormone so intimately involved in normal breast growth and differentiation would have nothing to do with breast malignancy. This paper examines the basis for a claim that PRL is a player in human breast cancer and that it is through an autocrine/paracrine pathway that this hormone acts.

Rodent mammary cancer

In the normal mammary gland, growth, development and differentiation is influenced by a variety of growth factors and hormones; the peptide hormone, prolactin, is considered a major player (Vonderhaar 1987). Functional differentiation of the gland as measured by induction of milk protein synthesis both in vivo and in vitro is dependent on PRL. Extensive growth and development of the alveolar cells within the lobules require PRL (Vonderhaar 1984, 1987). Progesterone increases the level of PRL receptors in the developing mammary gland (Nagasawa et al. 1985) and thus acts synergistically with PRL to induce cell growth.

Prolactin’s role in rodent mammary cancer has been established for some time (Welsch & Nagasawa 1977, Vonderhaar & Biswas 1987). Multiple pituitary isografts which secrete large amounts of PRL result in a significant increase in the incidence of spontaneous mammary tumors in mice (Muhlbuch & Boot 1959). Daily injections of PRL result in more mice with tumors compared with controls (Boot et al. 1962). In rats, there is a direct correlation between serum PRL levels and susceptibility of various rat strains to induction of mammary tumors by chemical carcinogens (Boyns et al. 1973). Tumors induced in rats by either nitrosomethyl urea or 7,12-dimethyl-benz[a]-anthracene are dependent on PRL for sustained growth (Mershon et al. 1995). In rodents there is a direct correlation between drug-induced hyperprolactinemia and

Human breast cancer

In contrast to the situation in rodent model systems, the function of PRL in the etiology and progression of human breast cancer is not clear. The discovery of extrapituitary PRL synthesis by breast tissue suggests that a re-examination of the role of PRL in human breast cancer is in order. For several years significant contradictory evidence in the literature on the role of this hormone in human disease has clouded the situation. However, there is significant evidence that PRL may play a role in human breast disease. In 1984, Holtkamp et al. (1984) reported that as many as 44% of patients with metastatic breast disease were hyperprolactinemic during the course of the disease. Several cases of breast carcinoma in association with prolactinoma have been reported (Strung et al. 1997). In a subset of women at risk for familial breast cancer, basal serum PRL levels were significantly elevated (Love & Rose 1985, Love et al. 1991). In addition, the circadian rhythm of PRL secretion from the pituitary differs between groups at high vs low risk of breast cancer (Haus et al. 1980), with no seasonal variations (Holdaway et al. 1997). In one study, hyperprolactinemia was found to be an important indicator of unfavorable prognosis in node-positive breast cancer patients, both when evaluated singly and in conjunction with steroid receptor status (Bhatavdekar et al. 1994). In another study (Patel et al. 1996), hyperprolactinemia and alterations in levels of p53 were associated with aggressiveness of the tumor, early disease relapse or metastases, and poor overall survival in patients with node-negative breast cancer. However, in contrast, a surgery-induced rise in PRL was paradoxically associated with a longer disease-free survival in operable breast carcinoma in patients both with or without axillary node involvement, despite the potential stimulation of cancer cell growth by the hormone (Lissoni et al. 1995). In addition, a significant decline in the serum level of insulin-like growth factor (IGF)-1 was associated with surgery-induced hyperprolactinemia (Barni et al. 1994), suggesting that the balance of specific hormones and growth factors may be a key etiological factor.

Prolactin effects in vitro

It has been known for nearly 20 years that more than 70% of human breast biopsies are positive for PRL receptors (Codegone et al. 1981, Peyrat et al. 1981, Bonnettre et al. 1982, L’Hermite-Baleriaux et al. 1984); approximately 80% of breast cancer cells in culture respond to PRL’s mitogenic signal when proper conditions of reduced serum or serum-free conditions are employed (Das & Vonderhaar 1997a). PRL itself can be detected in 60-85% of human breast cancer biopsies by immuno-histochemistry (Purnell et al. 1982, Agarwal et al. 1989). However, while the literature suggests that PRL levels may influence human breast cancer, there is no clear correlation between circulating PRL levels and the etiology or prognosis of the disease (Wang et al. 1986, Ingram et al. 1990, Love et al. 1991). Nor is there a clear clinical response when the secretion of pituitary PRL is impaired. In earlier studies, treating patients with PRL inhibiting ergot drugs in order to diminish the levels of circulating pituitary PRL resulted in no change in disease state over time (Henson et al. 1972, Pearson & Manni 1978). However, human growth hormone (hGH), which is also a lactogen, may have compensated for the diminished PRL levels. Therefore, another cohort of women with advanced breast cancer was given a combination therapy of bromocriptine, to diminish circulating PRL levels, and a somatostatin analog to block hGH action (Manni et al. 1989). As a result, circulating levels of PRL, detected by a single assay, were abolished nearly completely in 8 of 9 patients, whereas hGH levels were suppressed in 7 of 9 patients during treatment. However, the patients entering the study had been pretreated heavily with chemotherapeutic agents, thus making reliable assessment of overall antitumor effects difficult. Only one patient experienced disease stabilization. In a subsequent study, 4 of 6 patients with advanced breast cancer who had failed first and second line endocrine therapies experienced no evidence of disease progression for periods of up to 6 months when treated for extended periods of time with bromocriptine and the long-acting, superpotent somatostatin analog octreotide. While 24 h profiles of immunoreactive PRL, growth hormone (GH) and IGF-I in their serum were greatly reduced by these treatments, GH levels and diurnal peaks of bioreactive PRL were still apparent, although much reduced (Anderson et al. 1993).

Prolactin effects in vivo

Investigations in vitro using human breast tumor tissue and cells show clear responses to PRL in contrast to results collected in vivo. Among the responses are increased synthesis of DNA (Salih et al. 1972, Welsch et al. 1976, Peyrat et al. 1984, Calaf et al. 1986), protein (Burke & Gaffney 1978) and β-lactalbumin (Wilson et al. 1980), colony formation (Malarkey et al. 1983, Manni et al. 1986) and changes in shape, adhesion, lipid accumulation (Shiu & Paterson 1984) and estrogen receptor (ER) content (Shafie & Grantham 1981). Increased growth is observed when primary breast biopsy samples are grown in nude mice supplemented with PRL (McManus & Welsch 1984). Physiological levels of human PRL (hPRL) and hGH in culture increase the population doubling of primary breast tumor biopsies (Malarkey et al. 1983). In addition, we have shown that more than 80% of ER-
positive, as well as ER-negative, human breast cancer cell lines express mRNA for PRL receptors, bind PRL specifically with high affinity, and respond to PRL’s mitogenic signal (Biswas & Vonderhaar 1987, Das & Vonderhaar 1997a). Both T47D and MCF-7 human breast cancer cells respond to PRL’s growth signal when grown as solid tumors in nude mice (Vonderhaar & Biswas 1987). In the past, several reports have shown a lack of growth stimulation by PRL with these same human breast cancer cells. However, these studies were performed in the presence of high levels of fetal bovine serum, a rich source of lactogenic hormones (Biswas & Vonderhaar 1987). Only under serum-free conditions or in the presence of charcoal-stripped serum (CSS) can direct effects of PRL on growth of human breast cancer cells be achieved (Das et al. 1994, Lemus-Wilson et al. 1995, Das & Vonderhaar 1997a). We showed that PRL-stimulated growth of MCF-7 cells is greater in the presence of 1% CSS compared with 10% CSS or fetal bovine serum (Biswas & Vonderhaar 1987). Growth effects are seen at concentrations as low as 25 ng/ml hPRL; the maximal effect is observed at 100-250 ng/ml. Other lactogens such as hGH, human placental lactogen and ovine PRL also stimulate growth, but higher concentrations are required to achieve the same effect. Bovine PRL had no effect on the growth of these human breast cancer cells. Thus MCF-7 cells are more sensitive to the mitogenic effect of hPRL than to other lactogens or PRL from other species.

The effects of PRL on the growth of breast cancer cells is affected by the presence of other hormones and growth factors in the media. Growth of MCF-7 and ZR75.1 cells induced by hPRL is completely blocked by melatonin, the primary hormone from the pineal gland (Lemus-Wilson et al. 1995). When bovine PRL is added simultaneously with hPRL to the culture media the effect of hPRL on the growth of MCF-7 cells is completely abolished. As little as 50-100 ng/ml bovine PRL is able to block the hPRL-induced growth of these cells (Biswas & Vonderhaar 1987, Das & Vonderhaar 1997a). In contrast, bovine PRL is an effective mitogen and differentiating agent in normal mouse mammary cell lines in culture, suggesting that this hormone’s action as an antagonist of hPRL may be unique to human breast cancer cells.

### Synthesis and secretion of prolactin by breast cancer cells

In human breast cancer, the lack of clear clinical data to correlate with responses to PRL observed *in vitro* may be explained, in part, by the fact that breast cancer cells themselves are a source of extrapituitary PRL (Fig. 1). For many years, evidence in the literature has hinted at the possibility of an extrapituitary source of PRL. In one study, the levels of PRL in serum remained at 30-80% of the pre-surgical levels for as long as 10 months in breast cancer patients following total hypophysectomy (LaChellin et al. 1977). In a similar study by Manni et al. (1979), 128 of 156 breast cancer patients were determined to have a complete hypophysectomy as shown by the fact that their serum PRL was undetectable even after stimulation with chlorpromazine. However, 28 patients (18%) had low to normal levels of PRL which did not respond to stimulation. This latter group were categorized as ‘incomplete hypophysectomy’, but no confirming data were presented, leaving open the possibility that the PRL was from another source which would not necessarily be

### Table 1 Synthesis of PRL as a function of ER and PRL receptor (PRL-R) status.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>ER status</th>
<th>PRL-R status</th>
<th>PRL mRNA</th>
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<tr>
<td>ZR 75-1</td>
<td>+</td>
<td>+</td>
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<tr>
<td>T47D</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>MCF-7</td>
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<tr>
<td>T47Dco</td>
<td>-</td>
<td>+</td>
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<tr>
<td>MCF 10a</td>
<td>-</td>
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<tr>
<td>BT20</td>
<td>-</td>
<td>+</td>
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<tr>
<td>MDA MB-231</td>
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<tr>
<td>MDA MB-468</td>
<td>-</td>
<td>+</td>
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<tr>
<td>MDA MB-453</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
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<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>-</td>
<td>Not done</td>
<td>+</td>
</tr>
<tr>
<td>A1N4</td>
<td>-</td>
<td>+</td>
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subject to the same stimulatory mechanisms. In addition, low levels of circulating, bioactive PRL persisted in patients given bromocriptine plus a somatostatin analog to suppress pituitary hormone synthesis and activity (Anderson et al. 1993). Prolactin is generated by tumors as well as by a variety of normal tissues. Placenta is the richest source of extrapituitary PRL (Sinha 1995). The decidua is responsible for the high concentrations of PRL in human amniotic fluid. The brain, uterus, dermal fibroblasts and the immune system all produce PRL (Clevenger et al. 1990, Gellerson et al. 1994, Sinha 1995, Richards & Hartman 1996). Several laboratories, including our own, have shown that PRL is produced by both normal and malignant mammary epithelial cells and thus may be an autocrine/paracrine factor for this tissue. Steinmetz et al. (1993) showed by in situ hybridization that PRL gene transcripts are present in secretory mammary epithelial cells from pregnant rats. Northern analysis and the reverse transcriptase-polymerase chain reaction (RT-PCR) have provided evidence for local synthesis of PRL by mammary glands from lactating rats (Kurtz et al. 1993), goats and sheep (LeProvost et al. 1994). More recently, the presence of PRL mRNA has been demonstrated in human breast cancer cells lines in our laboratory (Ginsburg & Vonderhaar 1995) and in some primary human breast carcinomas (Clevenger et al. 1995a).

In addition, we demonstrated that bioactive PRL is synthesized by human breast cancer cells in culture and acts in an autocrine manner to stimulate cell proliferation. Growth of both T47Dco (ER-negative) and MCF-7 (ER-positive) human breast cancer cell lines was inhibited by 20-90% when cells were treated with monoclonal antibodies raised against human pituitary PRL (Ginsburg & Vonderhaar 1995). In addition, antisense RNA directed against the gene encoding for pituitary PRL significantly inhibited growth (>50%) of T47Dco cells (Ginsburg et al. 1997). Parallel cultures treated with a randomized antisense RNA sequence grew at the same rate as untreated controls. The presence of the mRNA for PRL in T47Dco and MCF-7 cells was confirmed by RT-PCR, followed by Northern analysis using pituitary hPRL cDNA as the probe (Ginsburg & Vonderhaar 1995). In total, 82% of all breast cancer cell lines tested (Table 1) contained mRNA for PRL (Ginsburg et al. 1997).

Using 35S-cysteine to metabolically label the T47Dco cells, the nature of the protein synthesized was assessed. Both cell extracts and conditioned media contain a 22 kDa protein precipitated by anti-hPRL monoclonal antibodies. Concentrated conditioned media prepared from T47Dco cells stimulated the growth of PRL-responsive Nb2 rat lymphoma cells to grow; these cells respond to picogram quantities of lactogens. The level of biological activity in the conditioned media is equivalent to 0.7 µg/ml (14.5 pg PRL/cell) pituitary PRL as measured by the Nb2 assay and is approximately 30% of the amount normally produced by the rat pituitary cell line, GH3 (Bancroft & Tashjian 1988).
By using a specific RIA for human pituitary PRL, 0.35 µg/ml PRL protein is detected. When the media are pretreated with an anti-pituitary PRL antibody, the activity in the conditioned media, like that of the human pituitary PRL, is abolished (Ginsburg & Vonderhaar 1995).

More than 80% of all breast cancer cell lines tested contain mRNA for PRL (Fig. 2). In this sample of cell lines, we found no correlation of ER status with the presence of PRL receptors or with the ability of the cells to synthesize PRL (Table 1). PRL gene transcripts were also found in tumors arising from MCF-7 cells implanted into nude mice (Ginsburg et al. 1997). Immunologically detectable PRL was present in 60-85% of human breast cancer biopsies, although the source of PRL is indeterminate (Purnell et al. 1982, Agarwal et al. 1989). More than 75% of primary breast cancer surgical samples also contain mRNA for PRL. In the majority of cases, the amount of mRNA for PRL and its receptors is significantly elevated in cancerous vs the adjacent, non-involved tissue from the same patient (Fields et al. 1993, Ginsburg et al. 1997). Similar results were reported by Touraine et al. (1998) who found PRL mRNA in all breast samples tested from 29 patients.

**Post translational modifications of PRL**

A PRL mRNA variant lacking exon 4 which arises from alternate splicing has been reported in rat brain (Emanuele et al. 1992). However, the mRNA for PRL isolated from late pregnant and lactating sheep and goat mammary glands differs from pituitary transcripts by only three mutations, two of which are silent (LeProvost et al. 1994).

Our recent data (E Ginsburg & BK Vonderhaar, unpublished observations) suggest at least 90% sequence identity between the PRL mRNA from the pituitary and from human breast cancer cells. Similar results have been reported in a variety of human breast cancer cell lines and neoplastic breast tissue samples (Shaw-Bruha et al. 1997). Because there is significant evidence that many diverse activities of PRL are modulated by different post-translational modifications, the key to the role of autocrine PRL in human breast cancer may lie therein. The biological activity and immunoreactivity of pituitary PRL are modified by phosphorylation and glycosylation (Markoff et al. 1988, Lewis et al. 1989). Phosphorylated PRL, present in murine, bovine and avian species (Walker 1994), has less activity compared with the nonphosphorylated form (Wicks & Brooks 1995). The phosphorylated form of PRL is unable to bind to the receptor due to conformational change in the hormone. The biological activity is restored by dephosphorylation.

Glycosylation may selectively down-regulate PRL action in target tissues (Hoffman et al. 1993). Glycosylated PRL, in the mammary casein synthesis and the Nb2 proliferation assays, has biological activity similar to or lower than that of the nonglycosylated form (Sinha et al. 1991, Pellegrini et al. 1992). Receptor binding activity and immunological cross-reactivity are greatly reduced as a result of glycosylation. Both glycosylated and nonglycosylated recombinant human PRL can be purified from the
murine C127 cell expression system. The 23 kDa non-glycosylated form of the PRL is 3-4 times more active in the Nb2 mitogenesis bioassay compared with the 25 kDa glycosylated form (Price et al. 1995). The changes in the ratio of glycosylated and nonglycosylated forms of PRL may explain physiologically diverse effects of PRL on target tissues (Young et al. 1990).

The nature of the post-translational alteration of PRL synthesized by the mammary gland and breast cancer cells is unknown. Our observations that a panel of monoclonal antibodies directed against pituitary PRL vary in their ability to recognize the PRL produced by breast cancer cells (Ginsburg & Vonderhaar 1995) suggests that there are marked differences in post-translational modifications between the product of the pituitary and that of the mammary gland.

**Cleaved prolactin and tumor angiogenesis**
The mammary gland cleaves PRL (Wong et al. 1986); three PRL species (25 kDa, 23 kDa and 14 kDa) are detected in rat tissue. Following bromocriptine treatment, the 25 kDa and 14 kDa forms remain, suggesting that they are the products of the mammary gland and that cleavage takes place (Lkhider et al. 1996). Experiments of normal mouse mammary tissue cleave PRL to yield two fragments (Baldocchi et al. 1995); the larger fragment of cleaved PRL, either 14 kDa or 16 kDa, has anti-angiogenic activity and inhibits the vascular endothelial growth factor (VEGF)-induced growth of capillary endothelial cells (Ferrara et al. 1991, D’Angelo et al. 1995). VEGF is essential for initial but not continued in vivo growth of human breast carcinoma cells (Yoshiji et al. 1997). Experiments from a transplantable rat tumor are unable to cleave PRL (Baldocchi et al. 1995).

**Regulation of expression of prolactin**
The regulatory mechanisms which control PRL synthesis and secretion by human breast cancer cells are not known. In the normal sheep and goat mammary gland, the PRL gene appears to be transcribed from the same proximal promoter used by the pituitary (LeProvost et al. 1994). However, other studies (Shaw-Bruha et al. 1997) suggest that, in human breast cancer cells, PRL synthesis is regulated by the distal promoter used by decidua and lymphocytes. Our data (E. Ginsburg & BK Vonderhaar, unpublished observations) show that one of the most effective regulators of mammary PRL synthesis is the hormone itself, suggesting an autoregulatory, feedback mechanism. Such a mechanism may explain the observation that treatment of lactating rats with bromocriptine results in a decrease in the PRL localization to the endocytic organelles and an increase in localization to the organelles associated with synthesis and exocytosis (Lkhider et al. 1996).

**Prolactin receptors**
PRL receptors belong to the cytokine hematopoietic family of receptors (Kelly et al. 1991). The members of this superfamily are single membrane-spanning receptors organized into three domains comprising an extracellular ligand binding domain, a hydrophobic transmembrane domain and an intracellular domain containing a proline-rich motif. The three different forms of the PRL receptor differ in their cytoplasmic domain. The long (90 kDa) and short (40 kDa) forms of the receptor are generated by differential splicing of a single gene and differ only in the length of the cytoplasmic domain (Kelly et al. 1993). The intermediate form of the receptor is a deletion mutant of the long form, lacking 198 amino acids in its cytoplasmic region. This form of the receptor is found in Nb2 rat lymphoma cells and is more sensitive to PRL compared with the other forms (Ali et al. 1992). It may be present in some human breast cancers (Clevenger et al. 1995a). Both the long and the intermediate forms of the PRL receptor transduce a differentiation signal as measured by induction of milk protein gene expression (Lesueur et al. 1991). All three forms promote mitosis (O’Neal & Yu Lee 1994, Das & Vonderhaar 1995).

Both normal and malignant mammary cells contain both long and short forms of the receptor. The receptors also exist as multiple charged forms. Dimerization of the membrane-associated receptor results from the interaction of PRL with its receptor (Goffin & Kelly 1997, Sakal et al. 1997). The physiological significance of homodimers vs heterodimers is unknown.

In human breast cancer, there is a clear correlation of ER and progesterone receptor (PR) status with a variety of disease parameters. However, because of the multiple size and charged forms of the PRL receptor, no similar clear correlation has been established for this hormone. PRL receptors have been demonstrated in over 70% of breast cancer biopsy samples using specific binding assays (Codecone et al. 1981, Peyrat et al. 1981, Bonneterre et al. 1982, L’Hermitte-Baleriaux et al. 1984). In our study (Mertani et al. 1998), virtually all breast cancer surgical samples were positive for PRL receptor mRNA by in situ hybridization, but the amount varied considerably. Quantitative estimation of receptor mRNA levels was regionally measured in areas corresponding to tumor cells or adipose cells in the same section. No correlation emerged according to the histological type of lesion, probably due to the large individual variation. Receptor immunoreactivity was detected in some scattered stromal cells but the labeling intensity was always weaker than for the neoplastic cells.
epithelial cells. In addition, the expression of mRNA as measured by RT-PCR in malignant tissue was always greater than in adjacent uninvolved tissue. Touraine et al. (1998) made a similar observation in tissue from 29 patients using quantitative PCR and immunohistochemistry. In our study (Mertani et al. 1998) the expression of PRL receptor occurred regardless of the ER or PR status. In a similar study, Reynolds et al. (1997) demonstrated by immunocytochemistry that >95% of breast cancers and >93% of normal breast tissues expressed the PRL receptor. They also found no association between the expression of PRL receptor and ER or PR status. These observations are in contrast to the report from Ormandy et al. (1997), who found that the level of PRL receptor expression in breast cancer cell lines was linearly related to that of the ER and PR status.

In human breast cancers, the ratio of long and short forms is unknown. The short form of the human PRL receptor has not yet been cloned. No message for the intermediate form of the receptor has been detected in our samples, in contrast to the report by Clevenger et al. (1995a).

**Prolactin mitogenic signaling pathways**

**PRL receptor-associated kinases**

Because PRL receptors do not have intrinsic kinase activity, they recruit several different kinases to transduce the mitogenic signal. JAK2, a member of the Janus family of kinases which is associated with the PRL receptor constitutively, is phosphorylated upon PRL binding in rat lymphoma (David et al. 1994, Rui et al. 1994), mouse mammary explants (Campbell et al. 1994) and murine lymphoid BAF-3 cells (Dusanter-Fourt et al. 1994). In murine lymphoid BAF-3 cells transfected with the long form of the PRL receptor, JAK1 is associated with the PRL receptor and is phosphorylated upon ligand binding (Dusanter-Fourt et al. 1994). The JAK kinase is transphosphorylated and activated as a result of receptor dimerization. Phosphorylation of the JAK proteins may be one of the earliest cellular events in response to the hormone which ultimately triggers a chain of events in the PRL signaling pathway.

The PRL receptor itself is phosphorylated within 1 min of PRL treatment, both in vivo in rabbit mammary gland and in vitro in Chinese hamster ovary (CHO) cells transfected with the long form of the PRL receptor cDNA (Waters et al. 1995). Binding of PRL to its receptors then induces phosphorylation of cytoplasmic transcription factors, mainly signal transducer and activator of transcription (STAT)-1, STAT-3 and STAT-5 (DaSilva et al. 1996). STAT-5 is activated for PRL’s differentiation signal (Wakao et al. 1994). Two different STAT-5 proteins have been isolated from mouse mammary tissue (STAT-5a; STAT-5b). Both of the transcription factors recognize GAS sequences. Their expression is concurrent during mammary gland development, increasing from the virgin state, reaching a maximum at day 16 of pregnancy and declining during lactation (Liu et al. 1995). STAT-5a is essential for full lobuloalveolar development and lactation, as demonstrated by use of knockout mice (Liu et al. 1996). The phosphorylation of STAT proteins has also been reported in Nb2 cells (David et al. 1994) and in normal mammary epithelial cells (HC11) (Wakao et al. 1994). We have reported activation of STAT-5 upon PRL treatment in T47D breast cancer cells (Das & Vonderhaar 1996b). DaSilva et al. (1996) also reported the phosphorylation of STAT-1, STAT-3 and STAT-5 in T47D cells within 15 min of PRL treatment. Activation of STAT proteins results in translocation of the transcription factors to the nucleus and activation of gene transcription (Darnell et al. 1994). STAT-1, and possibly STAT-3, is constitutively activated in breast cancer tissue (Watson & Miller 1995).

In hepatocytes of lactating rats in which the short form of the receptor is predominant, PRL induces the association of PRL receptors with pp60 c-src and activation of its tyrosine kinase activity (Berlanga et al. 1995). PRL stimulation in rat lymphoma Nb2 cells induces the association and activation of src family protein tyrosine kinase p59 fyn (Clevenger & Medaglia 1994) and also the guanine nucleotide releasing factor (GNRF)-vav (Clevenger et al. 1995b). In Nb2 cells, a protein tyrosine phosphatase, PTP1D, which is associated with the PRL receptor-JAK2 complex is essential for PRL signal transduction during induction of β-casein (Ali et al. 1996). Both tyrosine kinase inhibitors herbiyisin A and tyrphos-tin are able to decrease the expression of a β-lactoglobulin promoter/chloramphenicol acetyl transferase (CAT) con-struct by over 50% in CHO cells transfected with the rabbit PRL receptor. In addition, orthovanadate, an inhibitor of tyro-sine phosphatase, is able to substitute for PRL in inducing CAT responses in these cells (Daniel et al. 1996). This suggests an intricate role of both kinases and phosphatases during PRL signal transduction.

**The ras-mitogen activated protein kinase (MAPK) pathway**

A variety of growth factors and cytokines mediate proliferation by activating the ras-MAP kinase pathway of signal transduction. By measuring the guanine nucleotides bound to the protein, it is possible to demonstrate activation of ras p21 protein by PRL in a variety of cell systems (Erwin et al. 1995, Elberg et al. 1996). Raf-1, mitogen activated protein kinase kinase (MEK) and MAP kinase
are downstream kinases in the ras-MAPK pathway which are activated for mitosis induced by PRL. In Nb2 cells, PRL rapidly phosphorylates c-raf-1 (Clevenger et al. 1994) which is closely associated with the PRL receptor. We have reported the rapid and transient activation of these enzymes in both normal and breast cancer cells in response to 5 min PRL treatment (Das & Vonderhaar 1996a). Tyrosine kinase inhibitors block the MAP kinase activity and PRL-induced growth in these mammary cells (Das & Vonderhaar 1996a). Both the long and the short form of the PRL receptor are able to activate MAP kinase, with the activity reaching a peak within 5 min of PRL treatment (Das & Vonderhaar 1995). PRL also activates MAP kinase in rat liver, in vivo, and in Nb2 cells (Buckley et al. 1994, Piccoletti et al. 1994). Activation and nuclear translocation of protein kinase C (PKC) and MAP kinase have been reported during PRL-induced proliferation of Nb2 cells (Buckley et al. 1994, Ganguli et al. 1996). A preliminary report has shown PRL activation of 40S ribosomal protein S6 kinase (Carey & Liberti 1995), an enzyme downstream from MAP kinase in this signal cascade in Nb2 cells. The activation of S6 kinase reached its peak at 1.5-2 h after PRL treatment. Rapamycin, a specific inhibitor of S6 kinase, inhibited PRL-induced mitogenesis (Carey & Liberti 1995).

The PKC pathway
The PKC pathway of signal transduction may be involved in PRL-induced cell proliferation (Kiley et al. 1996). The phosphoinositide cycle is activated during PRL signal transduction, which then activates PKC. The phosphorylation and activation of phosphatidylinositol-3 (PI3)-kinase for signal transduction of PRL in Nb2 cells have been reported (Alsaakaf et al. 1996). Endogenously added phospholipase C, an enzyme that hydrolyzes t-0-phosphoinosit 4,5-diphosphate (PIP2) to D-myo-inositol triphosphate (IP3) and diacylglycerol, elicited PRL-like effects on ornithine decarboxylase activity and RNA synthesis in pregnant mouse mammary gland explants in culture (Rillema et al. 1983). In NOG-8 mammary cells, PRL activates and translocates PKC to the membranes within 5-10 min (Banerjee & Vonderhaar 1992). In addition, activation of PKC by PRL occurs in splenocytes, liver, hypothalamus and Nb2 cells (Buckley et al. 1988, Rillema et al. 1989). Waters and Rillema (1989) showed translocation of PKC upon PRL treatment in explants from mouse mammary glands. In addition activation of nuclear PKC by PRL has been reported in Nb2 cells (Buckley et al. 1988, Fan & Rillema 1993, Ganguli et al. 1996).

The SHC-Grb pathway
The JAK-SHC pathway is activated in 3T3-F442A cells through the GH receptor which is a member of the same receptor family as the PRL receptor (VanderKuur et al. 1995). The association of JAK2 with SHC occurs in a rapid and transient manner in Nb2 cells upon 15 min of PRL treatment (Erwin et al. 1995). In breast cells, both normal and malignant, we have shown that PRL can phosphorylate SHC proteins within 1 min of hormone treatment followed by association with the Grb2-son of sevenless (Sos) complex in these cells. Also, JAK2 is rapidly phosphorylated and associated with the SHC protein upon PRL binding to its receptors (Das & Vonderhaar 1996b). Thus for PRL signal transduction during the induction of the mitogenic response in human breast cancer cells, both the JAK-STAT and the SHC-ras-MAP kinase pathways are operative. Possible cross talk between the MAP kinase pathway and the JAK-STAT pathway has been suggested (David et al. 1995).

Early response genes
Cell growth results from modulation of early response genes and late response genes in the signaling pathways of a mitogen. Expression of a variety of early genes, including myc (Zabala & Garcia-Ruiz 1989), is induced by PRL in a number of systems. After 30 min PRL treatment in hepatocytes, there is a rapid induction of the expression of the proto-oncogenes c-fos, c-jun and c-src, even in the presence of cycloheximide (Berlanga et al. 1995). This suggests that PRL stimulates the expression of genes with AP-1 or serum response elements sequences in their promoter regions. An early response gene, Pim-1, which is a proto-oncogene encoding a conserved cytosolic serine/threonine protein kinase, is stimulated by PRL during mitogenesis of Nb2 and hematopoietic cells (Buckley et al. 1995). Peak expression occurs at 2-4 h of PRL treatment and is not affected by cycloheximide. Interferon regulatory factor-1 (IRF-1), another early activation gene, is induced over 20-fold in Nb2 cells by PRL. This gene is induced twice by PRL in a single cell cycle. First, it is induced during G1 at 30-60 min and then again during early S phase at 10-12 h of hormone treatment (Stevens et al. 1995). The second peak of IRF-1 mRNA expression in early S phase is dependent on the continuous presence of PRL throughout G1, and is correlated tightly with DNA synthesis and subsequent cell proliferation. The GAS site in the IRF-1 promoter is thought to act as a PRL responsive element that responds to the mitogenic signal of PRL in T cells. Its activation in breast cells has not been established.

Late response genes
Several cyclins, important regulators of cell cycle progression (Musgrove et al. 1996), are amplified in malignant cells compared with their normal counterpart. Cyclin D1 is among the most commonly overexpressed oncogenes in breast cancer. Cyclin D1 knockout mice are devoid of
PRL-dependent lobuloalveolar structures in the mature gland (Sicinski et al. 1995). In addition, following PRL stimulation of quiescent Nb2 cells, the cyclin D2 mRNA level increases in mid G1 phase and decreases sharply before S phase. The cyclin D3 level increases in late G1/early S phase and gradually decreases during S phase (Hosokawa et al. 1994). Further elucidation of the activation of specific late response genes should help clarify the distinguishing events in PRL-induced mitogenesis vs differentiation.

**Inhibition of prolactin action**

Because the mammary gland synthesizes its own PRL and the majority of human breast tumors contain PRL receptors, clinical approaches to controlling this disease in PRL-responsive tumors need to incorporate antagonists of PRL acting directly at the receptor level. Included among these are mutants of hGH and hPRL, some of which have been shown to have anti-prolactin activity *in vitro* under defined growth conditions (Fuh et al. 1992, 1993, Fan & Rillema 1993, Dattani et al. 1995, Fuh & Wells 1995.

**Figure 3** Inhibition of ligand-induced receptor dimerization. (A) Sequential hormone binding first through binding site 1, forming an inactive complex. The hormone then binds to a second receptor through site 2, which leads to dimerization and formation of an active complex. (B) Site 2 mutants prevent formation of active dimers. (C) Inhibitors, such as TAM, interfere with the ability of the hormone to bind to the receptor. Reprinted from Vonderhaar (1999).
Mode et al. 1996). These mutants usually involve that portion of the hormone identified as site 2 (Fig. 3) where amino acids with small side chain residues are replaced with amino acids carrying large side chains. Steric hindrance prevents binding of the mutant to the second receptor in the dimer and hence they act as hormone antagonists (Goffin & Kelly 1997).

**The anti-lactogen binding site (ALBS)**

In addition, tamoxifen (TAM), the first line of therapy in pre- and post-menopausal, ER-positive breast cancer patients, is also an anti-prolactin or anti-lactogen (Vonderhaar & Banerjee 1991). In some reports, TAM has been shown to be effective in a small number of ER-negative breast cancer patients (Hawkins et al. 1980, Furr & Jordan 1984, Nolvadex Adjuvant Trial Organization 1985). Work in vitro has also suggested that TAM can work through other ER-independent mechanisms. Frequently, estrogens can reverse TAM inhibition of cell proliferation in vitro when the anti-estrogen is used at low concentrations. However, the growth rates cannot be restored by estradiol in the presence of higher concentrations of the anti-estrogen (Sutherland et al. 1987). Growth of several ER-negative human breast cancer cell lines is inhibited by micromolar concentrations of TAM (Green et al. 1981, Chouvet et al. 1988, Das et al. 1994, Perry et al. 1995). Several other cellular proteins besides the ER may be directly affected by TAM (Vonderhaar & Banerjee 1991). Pollak et al. (1990) have suggested that, during TAM therapy, there is a decrease in circulating IGF-I which could account for the ER-independent response in some patients. Others have suggested that direct interaction of TAM with PKC may be responsible (O’Brien et al. 1985). We have shown that the anti-lactogenic activity of TAM results from interaction with the ALBS (Das et al. 1993) which is located on the PRL receptor.

The ALBS is a member of the family of high affinity membrane-associated binding sites called anti-estrogen binding sites or AEBS (Sutherland & Foo 1979, Sutherland et al. 1980) which have been identified in a variety of tissues (Gulino & Pasqualini 1982, Mehta et al. 1984). A TAM resistant clone of MCF-7 cells, RTx6, differs from the parent MCF-7 cells in having no AEBS, but is identical to the parent cells with respect to the ER content and affinity (Faye et al. 1987). These cells could not be inhibited by TAM, whereas MCF-7 cells were. Anti-estrogens, acting through the ALBS, inhibit the growth of PRL-responsive cells, even in the absence of ER (Das et al. 1994, Das & Vonderhaar 1997a). The ALBS, located on cellular membranes, binds TAM and related non-steroidal anti-estrogens with high affinity, but does not bind estrogens (Fig. 4) (Biswas & Vonderhaar 1991, Vonderhaar & Banerjee 1991). It is through the ALBS that TAM inhibits PRL-induced growth of ER-negative, Nb2 rat lymphoma cells (Biswas & Vonderhaar 1989); the effects are not reversed by estradiol. In addition, binding of PRL to particulate and solubilized microsomal membranes isolated from normal mammary glands of lactating mice is inhibited by direct addition of 10^{-10} M or greater concentrations of TAM.
concentrations of TAM to the binding assays (Biswas & Von der Haar 1991); estradiol did not have this effect. Maximal inhibition of PRL binding by TAM is observed in the light microsomes that contain the plasma membranes.

Characterization of the relationship of the ALBS to the PRL receptor (Das et al. 1993), showed that TAM decreases the number of binding sites without changing the receptor’s affinity for PRL. TAM acts by inhibiting the binding of PRL to its receptor (Fig. 3), rather than promoting dissociation of the hormone-receptor complex. The PRL receptor and the ALBS co-purify on either PRL-Sepharose or TAM-Sepharose affinity columns. Both are recognized by the anti-PRL receptor monoclonal antibody B6.2 (Das et al. 1993). ER-negative T47D cells bind lactogenic hormones specifically and this binding is inhibited by 30-50% when 10^{-11} M TAM is added directly to the whole cell binding reaction (Das et al. 1994). At 10^{-9} M, TAM inhibits PRL binding by 70-90% with complete inhibition at 10^{-7} M TAM. Subsequently, PRL-induced growth for both cell lines is inhibited. In the ER-negative mouse mammary epithelial cell line, NOG-8, TAM inhibits PRL binding and at concentrations as low as 10^{-9} M rapidly inhibits PRL signal transduction through Raf-1, MEK and MAP kinase. This, in turn, leads to inhibition of PRL-induced growth of these PRL-responsive mammmary cells (Das & Von der Haar 1997b). Thus, we conclude that the ALBS is on the PRL receptor, and that TAM and other related, non-steroidal, triphenylethylene anti-estrogens may inhibit growth of ER-negative human breast cancer cells in vitro through this mechanism.

Conclusion

The ability of PRL to stimulate growth of human breast cancer cells in culture, coupled with the presence of active receptors for PRL on the majority of breast carcinomas, suggests that this peptide hormone is an active player in this disease. Understanding its role is complicated by the fact that human breast cancer cells synthesize and secrete significant amounts of biologically active PRL. These data suggest that clinically useful reagents should be sought which act at the level of the target tissue. The drug tamoxifen, in addition to its action as an anti-estrogen, is also an anti-lactogen and hence may be a useful tool for understanding the potential mechanism of action of PRL in tumors which are PRL receptor-positive even if they are ER-negative.

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