Progesterone metabolites in breast cancer

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Abstract

In the 70 years since progesterone (P) was identified in corpus luteum extracts, its metabolism has been examined extensively in many tissues and cell lines from numerous species. In addition to the reproductive tissues and adrenals, every other tissue that has been investigated appears to have one or more P-metabolizing enzyme, each of which is specific for a particular site on the P molecule. In the past, the actions of the P metabolizing enzymes generally have been equated to a means of reducing the P concentration in the tissue microenvironment, and the products have been dismissed as inactive waste metabolites. In human breast tissues and cell lines, the following P-metabolizing enzymes have been identified: 5α-reductase, 3α-hydroxysteroid oxidoreductase (3α-HSO), 3β-HSO, 20α-HSO, and 6α-hydroxylation. Rather than providing diverse pathways for inactivating and controlling the concentration of P in breast tissue microenvironments, it is proposed that the enzymes act directly on P to produce two types of autocrines/paracrines with opposing regulatory roles in breast cancer. Evidence is reviewed which shows that P is directly converted to the 4-pregnenes, 3α-hydroxy-4-pregnen-20-one (3α-dihydroprogesterone; 3αHP) and 20α-dihydro-progesterone (20αHP), by the actions of 3α-HSO and 20α-HSO respectively and to the 5α-pregnane, 5α-pregnane-3,20-dione(5α-dihydroprogesterone; 5αP), by the irreversible action of 5α-reductase. In vitro studies on a number of breast cell lines indicate that 3αHP promotes normalcy by downregulating cell proliferation and detachment, whereas 5αP promotes mitogenesis and metastasis by stimulating cell proliferation and detachment. The hormones bind to novel, separate, and specific plasma membrane-based receptors and influence opposing actions on mitosis, apoptosis, and cytoskeletal and adhesion plaque molecules via cell signaling pathways. In normal tissue, the ratio of 4-pregnenes:5α-pregnanes is high because of high P 3α- and 20α-HSO activities/expression and low P 5α-reductase activity/expression. In breast tumor tissue and tumorigenic cell lines, the ratio is reversed in favor of the 5α-pregnanes because of altered P-metabolizing enzyme activities/expression. The evidence suggests that the promotion of breast cancer is related to changes in in situ concentrations of cancer-inhibiting and -promoting P metabolites. Current estrogen-based theories and therapies apply to only a fraction of all breast cancers; the majority (about two-thirds) of breast cancer cases are estrogen-insensitive and have lacked endocrine explanations. As the P metabolites, 5αP and 3αHP, have been shown to act with equal efficacy on all breast cell lines tested, regardless of their tumorigenicity, estrogen sensitivity, and estrogen receptor/progesterone receptor status, it is proposed that they offer a new hormonal basis for all forms of breast cancer. New diagnostic and therapeutic possibilities for breast cancer progression, control, regression, and prevention are suggested.

Introduction

The name ‘progesterone’ was first adopted in 1935 (Allen 1970), shortly after it was isolated from corpus luteum extracts (Allen & Corner 1929), purified (Allen 1930, Slotta et al. 1934), and structurally identified (Butenandt 1934). In the 70 years since its discovery, nearly 100,000 papers have been published dealing with progesterone P; (4-pregnen-3,20-dione) on many levels. As the name implies, its main actions have been linked primarily to human female reproductive aspects involving the uterine changes associated with the menstrual cycle and gestation. However, P is now known to influence (directly or indirectly) many other tissues and facets of regulatory physiology and endocrinology, including those of the mammary glands. Soon after its discovery, the metabolism of
P began to be investigated, primarily with the aim of determining its route of inactivation. It has become apparent that many tissues have P-metabolizing enzymes, which can modify different parts of the molecule. Although the resulting metabolites have been shown, in some tissues, to be active molecules in and of themselves, for the most part there has been a reluctance to accept them as anything other than waste products, with their formation as a means of decreasing the local P concentrations.

PR has long been linked to the proliferative changes in the normal breast, but its role in breast cancer is unclear. Recent studies have provided evidence that P metabolites formed in breast tissue have regulatory functions with respect to breast cancer that may previously have been attributed to P. We first suggested (Wiebe et al. 2000) that the P metabolites produced within breast tissues might be independently active hormones functioning as cancer-promoting or -inhibiting regulatory agents. By this hypothesis, the maintenance of normalcy or progression to neoplasia would depend on the ratios of pro- to anticancer P metabolites in the local breast tissue microenvironment.

The aim of this review is to summarize observations which indicate that most (if not all) tissues/cells may have some capacity to convert P and that mammary tissue in particular has enzymes which catalyze the direct conversion of P to two classes of active metabolites. Evidence is reviewed that these P metabolites function as independent pro- or anti-cancer autocrine/paracrine hormones that regulate cell proliferation, adhesion, apoptosis and cytoskeletal, and other cell status molecules via novel receptors located in the cell membrane and intrinsically linked to cell signaling pathways. Current endocrine therapies are based on suppressing estrogen levels or inhibiting its actions. Unfortunately, only a fraction of all breast cancer patients respond to this estrogen-based therapy and the response is only temporary (McGuire 1987). As the breast tissue P metabolites act on breast cell lines regardless of their tumorigenicity, estrogen sensitivity and estrogen receptor (ER) and progesterone receptor (PR) status, they are suggested to provide a new endocrine-based explanation for progression to the various forms of breast cancer as well as for the maintenance of normalcy in breast tissues. Based on the findings, it is proposed that in breast tissue P serves as a precursor for active steroid hormones whose relative concentrations determine the levels of mitogenic, apoptotic, and metastatic activities locally within the tissue.

**Progestosterone is metabolized by many tissues**

Soon after its identification, a large number of studies followed to determine the metabolism of P. In the early decades, many workers in the field identified and measured urinary metabolites of P with the aim of ascertaining how the body inactivated this progestagen. By 1954, almost 100 naturally occurring steroids had been isolated from tissue and urinary sources (Dorfman 1954). The urinary P derivatives were assumed to result from metabolism in the liver and included 5β-pregnanediol (5β-pregnane-3α,20α-diol) and pregnanolone (5β-pregnane-3α,20α-dione) as well as the 5α-pregnanes, 5α-pregnane-3,20-dione (5αP), 5α-pregnane-3α,20α-dione (5αP), 5α-pregnane-3α,20β-dione (5αP), and 5α-pregnane-3α,20β-dione (5αP).

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Thus, many metabolism studies from a large number of tissues and various species had indicated that, in addition to the gonads and adrenals, perhaps most, if not all, tissues have some capacity to convert P to other products. The studies had demonstrated the presence in tissues and cells of a number of enzymes capable of converting P to a variety of products.

In terms of neoplasia, the presence of P-metabolizing enzymes had been demonstrated in rat testicular interstitial cell tumors (Chatani et al. 1990), androblastoma (Sertoli-Leydig cell tumor) (Stegner & Lisboa 1984), dimethylbenz(a)anthracene (DMBA)-induced rat mammary tumors (Mori et al. 1978, Mori & Tamaoki 1980, Eechaute et al. 1983), human endometrial carcinoma (Collins & Jewkes 1974, Pollow et al. 1975), human breast tissues (Lloyd 1979, Miller 1990), modified breast cancer cell lines (T47Dco) (Fennessey et al. 1986, Horwitz et al. 1986), and virally transformed adrenocortical cells (Wiebe et al. 1987). Although selective differences in P-metabolizing enzyme activities between normal and tumor tissues were noted in some of these studies, they were not linked to any potential effects of the metabolites themselves on cancer induction or promotion prior to our studies (Wiebe et al. 2000).
Progesterone metabolism in breast tissues and breast cell lines

P was known to be involved in normal breast development as well as in the proliferative changes that occur during the menstrual cycle, pregnancy, and lactation (Going et al. 1988, Potten et al. 1988). However, its direct role in mammary cancer was not clear (McGuire & Horwitz 1977, King 1993) and a number of studies provided conflicting results. Some reports indicated stimulation (Anderson et al. 1989), while others observed regression of, or no effect on, human tumors (Horwitz et al. 1985, Santen et al. 1990) resulting from treatment with P or synthetic progestins. Similarly, in other species such as rodents (Jabara 1967, Welsh 1982, Luo et al. 1997) and dogs (Segaloff 1975, Mol et al. 1996), progestins were shown to either stimulate or inhibit tumor growth. In vitro studies of the effects of progestins on human breast cancer cell lines likewise showed either stimulation or inhibition of cell proliferation and cell cycle progression (Braunsberg et al. 1987, Clark & Sutherland 1990, Cappelletti et al. 1995, King 1993, Pike et al. 1993, Musgrove & Sutherland 1994, Clarke et al. 1994, Groshong et al. 1997).

The conflicting results regarding the role of P in breast cancer, in addition to the lack of evidence that tumor progression could be substantially related to changes in in situ P levels, led us to speculate about the potential importance of further metabolism of steroids occurring locally within the tumor and its adjacent host tissue. This led us to hypothesize that P may be converted within breast tissue into several types of metabolites, some of which stimulate while others inhibit cell proliferation and tumorigenesis. By this hypothesis, P would serve as a precursor (or pro-hormone) and the metabolites as the active hormones in regulating breast cancer. The state or progression of mammary tumors could then depend on the ratio of cancer-promoting to cancer-inhibiting steroid compounds. If such P metabolites could be shown to exist, they might provide an alternate or additional endocrine explanation for the estrogen-sensitive and -insensitive breast carcinomas as well as for normalcy of breast tissues.

Breast tissues and breast cell lines convert progesterone to 5α-pregnanes and 4-pregnenes

To test the hypothesis, studies were conducted to determine the capacity of tumor and surrounding normal (nontumorous) breast tissues to metabolize [14C]P. The paired tissue specimens came from premenopausal, menopausal and postmenopausal women with various subtypes and grades of infiltrating duct carcinomas and included tissues that were estrogen-receptor (ER) and progesterone-receptor (P) negative and/or positive (Wiebe et al. 2000). All the breast biopsies examined converted [14C]P into at least ten different metabolites that could be grouped into two structurally different classes of steroids (illustrated in Fig. 2): those with a delta-4 double bond in ring A (the 4-pregnenes) and those that are 5α-reduced (the 5α-pregnanes). Reduction of P to 5α-pregnanes is catalyzed by 5α-reductase and the direct 5α-reduced metabolite of P is 5α-pregnane-3,20-dione (5αP). The 5α-reductase reaction is irreversible, but 5αP can in turn be altered to 3- and 20-hydroxy pregnanes by the reversible actions of 3α-HSO, 3β-HSO, and 20α-HSO (Fig. 2).

The two 4-pregnenes resulting from direct P conversion are 4-pregnen-3α-ol-20-one (3αHP) and 4-pregnen-20α-ol-3-one (20αHP), catalyzed by the actions of 3α-HSO and 20α-HSO respectively (Fig. 2).

Figure 2 Progesterone conversion to 4-pregnen and 5α-pregnan metabolites by human breast tissues and cell lines. Note that 5α-reductase reaction is not reversible (see text for details; modified from Wiebe et al. 2005).
The 4-pregnenes can be further reversibly converted to 4-pregnene-3α(3β),20α-diol. The same metabolic pathways were subsequently demonstrated in four different breast cell lines (Wiebe & Lewis 2003) and had been previously identified in a number of tissues, including gonads, pituitary, and hypothalamus (Wiebe 1997). In addition, in the human breast cell lines, the final major product was 5α-pregnane-3β,6α-diol-20-one, indicating the presence of 6α-hydroxylase, an enzyme that was also present in tissues at minor activity levels. Thus, the P-metabolizing enzyme activities identified in human breast tissues and cell lines were: 5α-reductase, 3α-HSO, 3β-HSO, 20α-HSO, and 6α-hydroxylase (Fig. 2).

Changes in progesterone metabolite ratios and metabolizing enzyme activities

Although both normal (nontumorous) and tumorous breast tissues converted P to the two classes of metabolites, there were significant quantitative differences. In normal breast tissue, conversion to 4-pregnenes greatly exceeded the conversion to 5α-pregnenes, whereas in tumorous tissue, conversion to 5α-pregnenes greatly exceeded that to 4-pregnenes (Fig. 3a). The differences in amounts of 5α-pregnenes and 4-pregnenes were mainly due to changes in the amounts of 5αP and 3αHP (Fig. 3b) and the ratio of 5αP:3αHP was nearly 30-fold higher in tumorous than in normal breast tissues. The results indicated that P 5α-reductase activity is significantly higher, whereas P 3α-HSO and 20α-HSO activities are significantly lower in tumor than in normal tissues (Wiebe et al. 2000). Earlier studies with cell-free homogenates of breast tissues (Lloyd 1979, Miller 1990) and chemically induced rat mammary tumors (Mori et al. 1978) had also shown higher 5α-reductase and lower 20α-HSO activities in tumors than in normal glands.

Confirmation of a shift in actual amounts of P metabolites in the breast microenvironment has been provided, in part, by measurements of 5αP and 3αHP levels in breast tissue and nipple aspirate fluids (J P Wiebe, E Sauter & G Zhang unpublished results). The amounts of 5αP and 3αHP in a paired tissue sample, determined by gas chromatography–mass spectrometry, showed that levels (ng/mg protein) were 15.5 and 4.3 for 5αP and 5.5 and 12.7 for 3αHP respectively in the tumor and adjacent nontumor portion, confirming a higher 5αP:3αHP ratio in the tumor portion of the breast. An indication of the molar concentrations of P and the metabolites, 5αP and 3αHP, in breast microenvironment was obtained by RIA measurements of breast nipple aspirate fluids from four tumorous breasts (Table 1). Of note is that the concentrations in the aspirate fluid are high, being in the micromolar range. Although the values for 5αP varied considerably (perhaps due to the lack of specificity of the 5αP antibody), on average the levels of 5αP were higher than the levels of 3αHP; the concentrations of 5αP were 5.23 ± 2.51 μM and those of 3αHP were 1.03 ± 0.08 μM. The differences in levels suggest active metabolism of the
locally available P (also present at micromolar concentrations) and the ability of the cells to alter the microenvironment in terms of the P metabolites.

To determine if breast cell lines exhibit differences in direction of P metabolism related to tumorigenicity, estrogen response and/or ER/P status, four breast cell lines with varying characteristics were used (Wiebe & Lewis 2003). Three of the cell lines (MCF-7, MDA-MB-231, T47D) are known to be tumorigenic in immunodeficient mice (Anderson et al. 1984, Soto et al. 1986); among these, MCF-7 and T47D cells are ER/P-positive (Horwitz et al. 1975) and estrogen-dependent for tumorigenicity, whereas MDA-MB-231 cells are ER/P-negative and develop tumors spontaneously without estrogen. The fourth cell line, MCF-10A, is ER/P-negative and considered to be nontumorigenic (Soule et al. 1990). The results showed that production of 5α-pregnanes was higher and that of 4-pregnenes was lower in tumorigenic (e.g. MCF-7) than in nontumorigenic (e.g. MCF-10A) cells (Fig. 3c), while differences in ER/P status did not appear to play a role (Wiebe & Lewis 2003). The 5α-pregnane-to-4-pregnen ratios were 7- to 20-fold higher in the tumorigenic than in the nontumorigenic cell lines, providing essentially the same pattern of results as for the tissues.

Overall, the metabolism studies showed that the altered direction in P metabolism, and hence in metabolite ratios, was due to significantly elevated 5α-reductase and depressed 3α- and 20α-HSO activities in breast tumor tissues and tumorigenic cells. It appeared, therefore, that changes in P-metabolizing enzyme activities might be related to the shift toward mammary cell tumorigenicity and neoplasia. The changes in enzyme activity might reasonably be expected to be due to changes in expression of the enzyme genes.

Changes in expression of progesterone-metabolizing enzymes

The above metabolic studies and in vitro enzyme kinetics studies showed that the activity of 5α-reductase is higher, whereas that of the 3α(20α)-HSOs is lower in tumor tissue and tumorigenic breast cell lines than in normal breast tissue and cell lines. Several factors can account for changes in enzyme activity. In vivo, changes in enzyme activity can result from changes in levels of the enzyme due to changes in expression of the mRNA coding for the enzyme, or from changes in the milieu in which the enzyme operates (such as temperature and pH, and concentrations of cofactors, substrates, products, competitors, ions, phospholipids, and other molecules). In in vitro experiments, the milieu is carefully controlled to be identical between incubations, and therefore, observed differences can be more easily ascribed to differences in enzyme amounts.

To determine if the differences in P-metabolizing enzyme activities between normal and carcinoma tissues/cells could be attributed to changes in enzyme mRNA expression, reverse transcriptase (RT)-PCR studies were carried out on breast tissues and cells lines. RT-PCR analyses on tissues from 38 patients showed significantly higher levels of expression of 5α-reductase type 1 (SRD5A1) and 5α-reductase type 2 (SRD5A2) mRNA and significantly lower levels of expression of the 3α-HSO type 2 (AKR1C3), 3α-HSO type 3 (AKR1C2) and 20α-HSO (AKR1C1) mRNAs in the tumor tissues than in the normal tissues (Lewis et al. 2004) (Fig. 4a). These results were similar to those from enzyme mRNA expression studies on breast cell lines (Wiebe & Lewis 2003), which showed higher 5α-reductase and lower HSO gene expressions in tumorigenic than in nontumorigenic cell lines (Fig. 4b). Other reports also indicate lower HSO mRNA expression levels in tumor than in normal portions of breast (Ji et al. 2004) and prostate tissues (Ji et al. 2003).

Overall, the enzyme activity and expression studies strongly suggest that 5α-reductase stimulation and 3α- and 20α-HSO suppression are associated with the transition from normalcy to cancer of the breast. It is tempting to speculate that factors in the mammary

<table>
<thead>
<tr>
<th>Specimen</th>
<th>5αP (ng/μl)</th>
<th>3αHP (ng/μl)</th>
<th>Progesterone (ng/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.10 (3.48)</td>
<td>0.38 (1.2)</td>
<td>3.10 (9.81)</td>
</tr>
<tr>
<td>2</td>
<td>1.22 (3.86)</td>
<td>0.36 (1.14)</td>
<td>1.72 (5.48)</td>
</tr>
<tr>
<td>3</td>
<td>0.33 (1.04)</td>
<td>0.30 (0.95)</td>
<td>5.56 (17.7)</td>
</tr>
<tr>
<td>4</td>
<td>3.96 (12.53)</td>
<td>0.26 (0.82)</td>
<td>1.70 (5.41)</td>
</tr>
<tr>
<td>Mean ± S.E.M.</td>
<td>1.65 ± 0.79</td>
<td>0.33 ± 0.03</td>
<td>3.02 ± 0.91</td>
</tr>
</tbody>
</table>

Table 1 Levels of 5αP, 3αHP, and progesterone in breast nipple aspirate fluid samples from tumorous breasts.

*Breast nipple aspirate fluid samples (provided by Dr E. Sauter, University of Missouri, Columbia, MO, USA), were extracted and steroids separated chromatographically and measured by RIAs by methods similar to those described (Wiebe et al. 1991).
tissue milieu may be responsible for causing these changes in P-metabolizing enzyme gene expression and that these changes may be responsible for the transition. Steroid enzyme activities and gene expression have been shown in several tissues to be influenced by factors such as peptide hormones, cytokines, and steroids. For instance, prolactin acts as a paracrine/autocrine mutagenic agent in mammary cells (Clevenger & Plank 1997, Das & Vonderhaar 1997, Schroeder et al. 2002) and inhibits 20α-HSO expression in corpora lutea (Zhong & Vonderhaar 1997). In mammary gland cells, cytokines have been shown to regulate activity and expression of 3β-HSO (Gingras et al. 1999) and 17β-HSO (Turgeon et al. 1998). The level of expression of 5α-reductase is up-regulated by estradiol and P in the uterus (Minjarez et al. 2001) and by 5α-dihydrotestosterone (DHT) in the prostate (Andersson et al. 1989, Ji et al. 2003). And the expression of 20α-HSO may be altered by P in corpora lutea (Sugino et al. 1997) and in endometrial cells (Nakajima et al. 2003). These examples suggest that the changes in P-metabolizing enzyme activity/expression that lead to higher ratios of 5α-pregnane:4-pregnene may be induced by an altered milieu within the breast. Identification of the factors that may be responsible for changes in P-metabolizing enzyme expression awaits future investigations.

The studies cited above provided evidence of selective changes in levels of enzyme activities/expression in P metabolites formed in breast carcinoma, but there was as yet no evidence that P metabolites exhibited regulatory functions related to cancer. Some of the same P metabolites had been identified as active regulatory molecules in other tissues and with respect to other processes. For example, 5α-pregnanes such as 5αP (Selye 1942), 5α-pregnan-3α-ol-20-one (Majewska et al. 1986, Kavaliers & Wiebe 1987), and 3αHP (Wiebe & Kavaliers 1988) elicited marked anesthetic or analgesic effects via mechanisms involving calcium channels, the γ-aminobutyric acid (GABA)–benzodiazepine–chloride complex and endogenous opioid systems. 20αHP elevated serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels in rats (Gilles & Karavolas 1981), whereas 3αHP selectively suppressed basal and LH-releasing hormone-stimulated FSH secretion from primary cultures of anterior pituitary cells (Wood & Wiebe 1989) by nongenomic mechanisms at the level of the gonadotrope membrane, protein kinase C cell signaling pathway, and intracellular Ca2+ mobilization (Dhanvantari & Wiebe 2000).
Cancer-related actions of the progesterone metabolites
Transformation of normal human cells into malignant cancers involves changes in, or deregulation of, a number of cell characteristics and processes (Hanahan & Weinberg 2000). Cardinal among these are: (a) proliferation rates, (b) cell-to-cell and cell-to-substrate adhesion, (c) cytoskeletal and adhesion molecules, (d) receptors that transduce growth regulating signals, and (e) mitogenic growth signaling pathways. A summary of the effects of the P metabolites on these parameters follows.

Effects of progesterone metabolites on cell proliferation, mitosis, and apoptosis
Uncontrolled cell proliferation is one of the hallmarks of cancer, and factors which affect cell proliferation rates are known to affect cancer rates (Cohen & Ellwein 1990, Pike et al. 1993, Hanahan & Weinberg 2000). Initial studies conducted on MCF-7 cells showed significant, but opposite, effects on cell proliferation; 3αHP inhibited whereas 5αP-stimulated proliferation dose-dependently between 10⁻⁹ and 10⁻⁶ M (Fig. 5a). In this concentration range, estradiol resulted in weak stimulation at 10⁻⁸ M and either no effects or slight inhibition at higher concentrations (Fig. 5a). Stimulation in cell numbers was also observed when cells were treated with other 5α-pregnanes, such as 5α-pregnan-3α-ol-20-one, 5α-pregnan-20α-ol-3-one, and 5α-pregnane-3α,20α-diol, whereas other 4-pregnenes such as 20α-HP and 4-pregnene-3α,20α-diol resulted in suppression of cell proliferation similar to that of 3αHP (Wiebe et al. 2000). Stimulation of cell proliferation with 5αP and inhibition with 3αHP were also observed in all other breast cell lines examined, whether ER/P-negative (MCF-10A, MDA-MB-231) or ER/P-positive (T47D, ZR-75-1) and whether requiring estrogen for tumorigenicity (MCF-7, T47D) or not (MDA-MB-231), or whether they are nontumorigenic (MCF-10A) (Wiebe et al. 2000, Pawlak et al. 2005, G Zhang & J P Wiebe, unpublished results).

Increases in cell numbers can result not only from increased rates of cell division, but also from decreases in rate of cell attrition via programmed cell death (apoptosis) (Thompson 1995). A balance of proliferation and apoptosis provides the homeostasis in normal tissues and alteration in this balance is postulated to set off a series of changes ultimately leading to malignancy. Studies on cell lines (Zhang et al. 2005, G Zhang & J P Wiebe, unpublished results) using several methods of evaluating apoptosis and proliferation/mitosis showed that 3αHP resulted in significant increases in apoptosis and decreases in mitosis, leading to significant decreases in total cell numbers. In

Figure 5 Effects of 5αP and 3αHP exposure (72 h) on proliferation (cell number) and adhesion (detachment) of MCF-7 and MCF-10A human breast cell lines. Values are presented as percent in relation to controls (0, C). In (a) and (b) cells were exposed to 0 or 10⁻⁹–10⁻⁶ M 5αP or 3αHP and results show highly significant dose-dependent effects. In (a) MCF-7 cells were also exposed to estradiol for comparison purposes. The effect of combining 5αP and 3αHP (each at 10⁻⁷ M) on proliferation and detachment of MCF-7 cells are shown in (c); different letters (a, b, and c) on bars denote significant differences (P<0.05) within proliferation or detachment results. (Modified from Wiebe et al. 2000, Wiebe & Muzia 2001, Pawlak et al. 2005.)
contrast, treatment with 5zP resulted in decreases in apoptosis and increases in mitosis. Thus, with respect to overall cell proliferation effects, the results indicated that the actions of 3zHP and 5zP are diametrically opposed and involve both cell division and cell death. The results correlated with the metabolism studies in that the levels of the proliferation-inducing hormone (5zP) were higher and those of the proliferation-suppressing hormone (3zHP) were lower in tumorous tissue and the reverse was true for normal tissue.

**Effects of progesterone metabolites on cell adhesion**

Cellular adhesion is a critical aspect of cancer biology. In culture conditions, normal cells of mesenchymal or epithelial origin generally depend on anchoring to a solid substratum for cell division. This dependence on support by solid substrates for cell proliferation is lessened as cells become neoplastic and metastatic (Raz 1988). Some time during the development of most types of human cancer, pioneer cells are spawned that are capable of moving out of the primary tumor masses and of traveling to distant sites where they may succeed in founding new colonies. It is these distant settlements of tumor cells — metastases — that are the cause of about 90% of human cancer deaths (Sporn 1996). The capability of escaping the primary tumor mass and colonizing new terrain involves a number of cellular changes, not the least of which are cell–cell and cell–substrate adhesion characteristics. To allow the initial escape, adhesion must be decreased and attachment severed.

To determine whether P metabolites might play a role in the acquisition of metastatic potential, their effects on cell adhesion were examined (Wiebe et al. 2000, Wiebe & Muzia 2001) by quantitative cell–substrate attachment and detachment assays that had been developed earlier (Dinsdale et al. 1992) for baby hamster kidney cells. The first tests were on MCF-7 cells and the results showed that 5zP caused significant dose-dependent decreases in attachment to, and increases in detachment from, the substratum (Fig. 5b). The opposite effect was observed with 3zHP, which promoted cell attachment and decreased cell detachment (Fig. 5b). Similar effects have also been demonstrated recently in MCF-10A, T47D, and MDA-MB-231 cells (Wiebe et al. 2004, Pawlak et al. 2005). The opposing actions of 5zP and 3zHP on both cell anchorage and proliferation strengthen the hypothesis that the direction of P metabolism in vivo toward higher 5z-pregnane and lower 4-pregnene concentrations could promote breast neoplasia and lead to malignancy.

**Proof of principle**

Confirmation of the hypothesis that the move from normalcy to neoplasia in breast cells is influenced by the in situ increase in the 5z-pregnane:4-pregnen ratio requires studies in which 5z-reductase activity is blocked, as well as paradigms where various concentrations of a 5z-pregnane and a 4-pregnene are used in combination and in various temporal sequences. We have used the 4-azasteroid dutasteride, a known inhibitor of 5z-reductase types 1 and 2 (Bramson et al. 1997) that has been employed in trials to inhibit the 5z-reduction of testosterone to DHT in men with benign prostate hyperplasia (Brown & Nuttal 2003, Clark et al. 2004) and prostate cancer (Andriole et al. 2004, Iczkowski et al. 2005). First, we demonstrated that in MCF-7 cells dutasteride at 10⁻⁶ M inhibited P conversion to 5z-pregnanes by > 95% and at the same time increased 4-pregnen production. Next, it was demonstrated that treatment of cells with P alone, without medium change for 72 h, resulted in significant conversion to 5z-pregnanes and concomitant increases in cell proliferation and detachment. These increases in proliferation and detachment were blocked in cells incubated with P plus dutasteride. In turn, the suppression by dutasteride was overridden by the addition of 5zP. The results are seen as providing proof of the principle that the effects on proliferation and adhesion were not due to P, but due to the 5z-reduced metabolites (Wiebe et al. 2006).

To confirm the hypothesis that the ratio of 5z-pregnanes:4-pregnenes is a determinant of the degree of cell proliferation and adhesion, detailed studies will need to be carried out using various concentrations of 3zHP and 5zP in combination and in various temporal sequences. Similar studies could also determine if the progression toward neoplasia can be impeded or even reversed by high 3zHP:5zP ratios, i.e. ratios of P metabolites that favor the 4-pregnenes. Data from studies in which cells were treated simultaneously with both 3zHP and 5zP show that the independent effects of the individual hormones on proliferation and adhesion are cancelled out when present in equal concentrations (Fig. 5c) (Pawlak et al. 2005) and support the view that the overall effects may depend on the relative concentration of each in the milieu.
Effects of progesterone metabolites on cytoskeletal and adhesion complexes

The transformations in morphology, replication, and adhesion during the transition from normal to cancerous cell have been shown to be accompanied by rearrangements of cytoskeletal and adhesion structures. The cytoskeletal organization differs between normal and cancerous cells (Ben-Ze’ev 1985, Holme 1990, Holth et al. 1998) and between high- and low-metastatic cells (Suzuki et al. 1998). For example, the level of organization of the actin cytoskeleton observed in normal cells (Bershadsky et al. 1995, Helige et al. 1997) is characterized by higher levels of polymerized actin, whereas transformation to the metastatic condition may be accompanied by disruption and/or visible disappearance of actin filaments (Suzuki et al. 1998). Similarly, vinculin, a protein that is associated with cell-to-cell and cell-to-substrate adhesion sites (Wilkins & Lin 1982, Luna & Hitt 1992, Humphries & Newham 1998), may show alterations. In normal cells, vinculin may be readily detected, while in highly malignant cell lines its organization may be significantly altered (Schliwa et al. 1984) or it may not be detected at all (Sadano et al. 1992), suggesting that depolymerization or suppression of vinculin expression may be closely related to progression of malignancy.

To determine the cellular sites of action of the proliferation- and detachment-promoting P metabolite, 5αP, its effects on MCF-7 cell morphology, F-actin expression, polymerization, and filament distribution, as well as vinculin expression and vinculin-containing adhesion plaque numbers, were examined by immunohistochemistry, morphometry, and western blotting (Wiebe & Muzia 2001). Figure 6a shows typical distribution of polymerized actin filaments and terminal vinculin molecules in a normal cell. Treatment of cells with 5αP resulted in dose-dependent decreases in vinculin-containing adhesion plaques and vinculin expression (Fig. 6b), as well as in polymerized actin stress fibers (Fig. 6c). Similar results were observed with MCF-10A, MDA-MB-231, and T47D breast cell lines (Wiebe et al. 2004), again confirming that the P metabolites appear to be able to target a variety of human breast cells. The results suggest that the observed decreases in adhesion and increases in cell proliferation following 5αP-treatment may be related to depolymerization of actin and decreased expression of vinculin.

Receptors for progesterone metabolites in human breast cells

Localization and characterization of progesterone metabolite receptors

The actions of hormonal steroids are considered to generally require complexing with specific-binding sites (receptors) on target cells. Therefore, an important step in elucidating the mechanisms of action of a regulatory hormone is the identification of such receptors. To identify potential binding sites for P metabolites in mammary cells, competition radioreceptor assays were

Figure 6 Examples of effects of 5αP on cytoskeletal and adhesion complex molecules in MCF-7 cells. (a) The arrangement of vinculin-containing adhesion plaques (red) and polymerized F-actin fibers (green) is shown in a control cell. The effects of 5αP (10^{-8} M) treatment result in marked reduction in vinculin expression (inset) and in number of vinculin-containing adhesion plaques (b), and depolymerization (insoluble to soluble change) of F-actin (c). (From Wiebe & Muzia, 2001 and unpublished results).
conducted on nuclear, cytosolic, and membrane fractions of MCF-7 and MCF-10A breast cell lines using $[^3\text{H}]$-labeled 5zP and 3zHP (Weiler & Wiebe 2000, Pawlak et al. 2005). The studies showed that binding of 5zP or 3zHP occurs in the plasma membrane fractions, but not in the nuclear or cytosolic compartments (Fig. 7a). Saturation and Scatchard analyses indicated separate high-specificity, high-affinity, low-capacity receptors for 5zP and 3zHP that are distinct from each other and from the well-studied nuclear/cytosolic P, estrogen, and androgen and corticosteroid receptors; binding of $[^3\text{H}]5zP$ or $[^3\text{H}]3zHP$ was not displaced by 200 to 500-fold concentrations of P, estradiol, androgens, corticosteroids, and other P metabolites. In turn, binding of $[^3\text{H}]$ or $[^3\text{H}]$estradiol to cytosolic or nuclear fractions was not displaced by excess 5zP or 3zHP. The binding studies showed that the criteria of high affinity, specificity, saturability, and association and dissociation kinetics required of receptor designation (Laduron 1984, Limbird 1996) were met. The studies thus provided the first demonstration of the existence of specific P metabolite receptors. Identifying the presence of distinct and separate receptors for 3zHP (3zHPR) and 5zP (5zPR) in human breast cells is important in light of the findings that the two P metabolites exert opposing actions with respect to cell proliferation and adhesion.

Regulation of progesterone metabolite receptor levels

Since the action mechanisms of hormonal steroids are generally initiated by the binding to specific receptors, the level of cellular response to steroids is limited not only by the local concentration of the hormone, but also by the receptor number (Vanderbilt et al. 1987, Webb et al. 1992). Due to the potential importance of 5zP in promoting breast cancer via the binding to its membrane-based receptors, the role of mitogenic (estradiol, 5zP) and anti-mitogenic (3zHP, 20zHP) endogenous steroids on 5zPR levels in a tumorigenic (MCF-7) and a nontumorigenic (MCF-10A) breast cell line were explored (Pawlak et al. 2005). Exposure of MCF-7 cells for 24 h to estradiol or 5zP resulted in significant dose-dependent increases in 5zPR levels (Fig. 7b), whereas 3zHP or 20zHP resulted in significant dose-dependent decreases in 5zPR levels (Fig. 7c). Treatment with two mitogenic (estradiol or 5zP) or two anti-mitogenic (3zHP or 20zHP) hormones resulted in additive effects on 5zPR numbers (Fig. 7b and c), whereas treatment with one mitogenic and one anti-mitogenic hormone abolished the mitogen-induced increases (Fig. 7d). In addition, preliminary experiments in which MCF-7 cells were exposed to 1.0 nM estradiol for 24 h showed a 60% decrease in 3zHPR numbers (Weiler & Wiebe 2000).

The nontumorigenic breast cell line, MCF-10A, was also shown to possess specific, high-affinity plasma membrane receptors for 5zP that are up-regulated by estradiol and 5zP and down-regulated by 3zHP (Pawlak et al. 2005). Estradiol binding was demonstrated in MCF-10A cell membrane fractions and may explain the estradiol action in these cells, which reportedly lack intracellular ER. In both MCF-7 and MCF-10A cells, the increases in 5zPR due to estradiol or 5zP and decreases due to 3zHP or 20zHP correlated with respective increases and decreases in cell proliferation as well as detachment (Pawlak et al. 2005), indicating the functional relevance of
alterations in 5αP concentrations. Together, the receptor results suggest that the putative tumorigenic actions of 5αP may be significantly augmented by the estradiol-induced increases in 5αP binding and decreases in 3αHP binding.

Role of progesterone metabolites in regulating ER levels

Estradiol can influence mitogenicity of ER-positive mammary cells and therefore the regulation of ER levels may be important for the progression of estrogen-dependent mammary neoplasias. Estradiol and P are known to play a role in modulating ER concentrations (Shyamala et al. 2002). To determine if P metabolites affect ER levels, MCF-7 cells were exposed for 24 h to 5αP, 3αHP, 20αHP, and estradiol, or combinations of these steroids, and ER concentrations were determined in cytosolic and nuclear fractions by specific-binding of [3H]estradiol (Pawlak & Wiebe 2005). Estradiol and 5αP resulted in significant dose-dependent increases, whereas 3αHP and 20αHP each resulted in dose-dependent decreases in total ER as well as inhibition of estradiol- or 5αP-induced ER levels. In combination, estradiol + 5αP or 3αHP + 20αHP resulted in additive increases or decreases respectively in ER numbers.

The results are the first to show that the pro- and anti-cancer P metabolites have also marked selective (up or down) regulatory effects on ER levels in ER-positive MCF-7 breast cancer cells. The suggested implications for breast cancer are that the stimulatory and inhibitory effects of 5αP and 3αHP respectively on cell replication and cell detachment might be significantly modified by exposure to estradiol, 4-pregnenes, and 5α-pregnanes and, in turn, that the P metabolites may significantly affect ER response in estrogen-targeted cells.

Effect of the progesterone metabolite, 5αP, on cell signaling pathways

The location of the receptors for 5αP and 3αHP on the cell membrane suggests involvement of nongenomic mechanisms of action via cell signaling pathways. Modes of action via plasma membrane-based binding sites and cell signaling pathways have been suggested for estradiol (Watson et al. 1999, Keshamouni et al. 2002, Purves-Tyson & Keast 2004, Simoncini et al. 2004), corticosteroids (Wehling 1997, Croxall et al. 2000), 3αHP (Dhanvantari & Wiebe 1994, Beck et al. 1997, Wiebe 1997), and neurosteroids such as the P metabolite, 5α-pregnan-3α-ol-20-one (allopregnanolone) (Majewska et al. 1986). Signaling pathways that control cell proliferation and adhesion involve the mitogen-activated protein kinase (MAPK) pathway and, in turn, deregulation of this Ras-Raf-MEK-MAPK cascade plays a central role in human cancer (Chang & Karin 2001, Pearson et al. 2001, Santen et al. 2002). Studies on serum-starved MCF-7 cells showed that treatment with 5αP for as briefly as 5 min resulted in significant, dose-dependent increases in activated (phosphorylated) MAPK (Erk1/2) (Wiebe et al. 2005, Cialucu & J P Wiebe, unpublished results). Treatment with the MEK inhibitor, PD98059, resulted in significant suppression of the 5αP-induced MAPK activation. Similarly, in concomitant cell proliferation ([3H]thymidine uptake) and detachment assays, 5αP resulted in significant increases in cell proliferation and detachment, whereas PD98059 significantly suppressed the 5αP-induced increases. The data suggest that the action of 5αP on breast cancer cells involves modulation of the MAPK signaling pathway. Whether other cell signaling pathways are involved or 5αP and 3αHP act via different pathways in promoting or inhibiting neoplasia in breast cells remain to be explored.

Implications of changes in progesterone 5α-reductase activity for androgen action in breast cancer

Although the majority of primary human breast cancers express androgen receptors, no direct association with any androgen and breast cancer growth and progression has been convincingly established (Bradlow & Sepkovic 2004). How might the up-regulation in 5α-reductase in neoplastic breast tissue influence androgen metabolism in the breast and in turn affect the role of transformed androgens in breast cancer?

Suzuki et al. (2001) have suggested that increased conversion of testosterone to DHT resulting from increased 5α-reductase activity should inhibit cancer cell proliferation in human breast carcinoma. However, studies with ZR-75-1 (Poulin et al. 1988, Birrell et al. 1995, Kandouz et al. 1999), T47-D (Birrell et al. 1995, Ortmann et al. 2002), MDA-MB-231 (Di Monaco et al. 1995, Ortmann et al. 2002), MFM-223 (Hackenberg et al. 1991), and CAMA-1 cells (Lapointe & Labrie 2001) and with DMBA-induced rat mammary tumors (Bocuzzi et al. 1995) have shown that both testosterone and DHT inhibit cell growth more or less to the same extent. This is in marked contrast to the actions of P metabolites, where the 5α-pregnanes stimulate and the 4-pregnenes inhibit cell proliferation. Also, 5α-reductase type 2 (SRD5A2), which catalyzes
reduction of testosterone to DHT in androgen-dependent tissues such as the prostate, is present in very low levels in breast tissue (Ji et al. 2004, Lewis et al. 2004) and human breast cancer cell lines (Wiebe & Lewis 2003). In breast tissue, 5α-reductase type 1 (SRD5A1) is predominant and it may be that P is a better substrate than testosterone for this isoenzyme. Overall, current evidence does not appear to support the notion that increased 5α-reductase activity/expression might significantly alter androgen influences on breast tumor growth.

Implications of progesterone-metabolizing enzymes for synthetic progestin-based contraceptives and hormone-replacement therapy drugs

The synthetic progestins used for contraception and hormone replacement therapy (HRT) do not behave like P in terms of their metabolism and probably not with respect to their actions at the level of the breast tissue microenvironment. As different formulations may exhibit marked differences in chemical structure, metabolism, and pharmacodynamic actions, it is not possible to generalize about them. The effects of the drugs at the level of the breast tissue will be governed by the molecular form and bioavailability, but unfortunately these are areas that remain unexplored.

At the outset, the level of metabolism may vary greatly, depending on whether the route of administration is oral, transdermal, subcutaneous, or intravaginally (Fotherby 1996). When taken orally, many drugs are readily metabolized in the gastrointestinal tract and/or liver and the degree and site of metabolism varies substantially between different compounds. Some contraceptive and HRT drugs (for example, desogestrel, norgestimate, mestranol, norethisterone acetate, and ethinylestradiol-3-methyl ether) are in fact prodrugs and are converted into their active metabolites when taken orally (Fotherby 1996, Henzl 2001). On the other hand, compounds like Nesterone must be administered parenterally due to their rapid hepatic metabolism and apparent inactivation (Sitruk-Ware 2004). The different formulations also exhibit great variation in level of binding to serum proteins (Kuhl 1996, Hammond et al. 2003), potential action via estrogen, androgen, P, and corticosteroid receptor binding and consequent androgenic, estrogenic, and progestational potency, and actions on enzymes (Kuhl 1996). To ascertain the possible role of the contraceptive and HRT drugs in breast cancer regulation via the P metabolites, it will be necessary to measure their levels and composition in the breast microenvironment to determine their effects on P metabolism in breast tissue and/or cell lines and to establish whether the P-metabolizing enzymes can further alter the drugs to pro- or anti-cancer moieties.

Summary, significance, and future prospects

Mammary gland cells show cyclicity and respond to steroid hormones. Normal breast tissue goes through cycles of imbalance between proliferation and apoptosis during menstrual periodicity, pregnancy, and lactation, but regularly corrects these temporary imbalances. In cancer, changes have occurred such that overall increases in cell numbers continue and result in the development of tumors. The normal changes are believed to be due to the changes in concentration of the ovarian hormones, estradiol and P. Since estrogens have been shown to increase proliferation in some cells, and because about one-third of breast cancer patients show some responses to anti-estrogen therapies, estrogens have been considered the primary hormonal cause of breast cancer. In time, however, estrogen-sensitive neoplasms become unresponsive and the patients experience relapse. Overall, this means the existence of an overwhelming majority of breast cancers for which the current estrogens based explanations and therapies are inadequate. Since P appears to be involved in the normal cyclical changes, it too has been implicated in breast neoplasia, but its role has been unclear and no specific categories of breast cancers have been shown to respond unambiguously to P or to anti-progestins. The end result is that for the majority of breast cancers, current estradiol/P-based explanations are inadequate and therapies ultimately ineffective. Moreover, estradiol and P do not provide hormone-based explanations for those breast tissues that do not become cancerous.

The P metabolites, produced within breast tissues, are put forward as potential candidates that could up- or down-regulate mitogenic and metastatic processes in various (perhaps all) mammary tissues, resulting in maintenance of normalcy or in progression to cancer. The suggestion is based on the following lines of evidence and summarized in Fig. 8: (1) Breast tissue, like many other tissues, has a number of enzymes capable of catalyzing the conversion of P to various metabolites, which can be grossly grouped into 5α-pregnanes and 4-pregnenes. (2) In breast tumor tissue and tumorigenic cell lines, 5α-reductase activity and mRNA expression are significantly higher, whereas 3α- and 20α-HSO activities and mRNA expression are significantly lower than in normal breast tissue and

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nontumorigenic cells. (3) The result is that in para- and intra-cell environments of localized regions of the breast, the levels of 5α-pregnanes such as 5αP are increased, whereas those of the 4-pregnenes like 3αHP are decreased. (4) Studies using various breast cell lines have shown that 5αP and 3αHP have opposing actions in terms of cell proliferation and adhesion; 5αP stimulates cell proliferation (through increased mitosis and decreased apoptosis) and cell detachment, whereas 3αHP suppresses cell proliferation (through decreased mitosis and increased apoptosis) and cell detachment. (5) Separate mechanisms of action of 5αP and 3αHP are proposed, involving binding to separate, specific, and novel membrane receptors that are up- or down-regulated by estradiol and the P metabolites and that are linked to cell signaling pathways which transcribe different effects on cytoskeletal and adhesion molecules. (6) Based on the in vitro results, the paracrine/autocrine functions of 5αP are cancer-promoting and those of 3αHP are cancer-inhibiting. Changes from normal status to progression through increasing degrees of neoplasia are determined by changes in the relative concentrations of the pro- and anti-cancer hormones in the microenvironment. (7) As the P metabolites affect cell lines with various characteristic (ER/P-positive or -negative, tumorogenic or nontumorogenic, estrogen-sensitive or -insensitive), it is suggested that they may be general determinants of normalcy or cancer of the human mammary gland (Wiebe 2005). They may thus provide a new endocrine basis for the majority of human breast cancers that do not respond to ER-based therapy and also an alternate one for those that do.

The work on the potential role of P metabolites in promoting normalcy or cancer of the breast is in its infancy and a number of issues need to be addressed. First, all the observations summarized in this review about the effects/actions of the P metabolites were made in vitro on breast cell lines in culture.
To substantiate the hypothesis that the P metabolites play a role in mammary cancer, it is necessary to demonstrate their effects in vivo. Such experiments would test the P metabolites, 5αP and 3αHP, for their independent and combined effects on promotion or inhibition of growth of mammary tumors in mouse models resulting from human cell line inoculates and/or derived spontaneously or by chemical induction. Encouraging evidence that P metabolites can have similar effects in vivo and in vitro has come from a pilot experiment conducted by Drs R Schillaci and P Elizalde (NRC, Buenos Aires). They showed (personal communication) that C4HD murine cells inoculated into BALB/c mice developed into substantial palpable tumors if treated with 5αP (40 mg depot). Tumor growth rate was about the same (or slightly higher) with 5αP as with an equivalent dose of medroxyprogesterone acetate, a known tumor inducer in this model (Lanari et al. 1986). Secondly, in terms of potentially preventing, suppressing, or regressing breast tumors, more attention (both in vivo and in vitro) needs to be directed at the presumptive anti-cancer P metabolite, 3αHP, as well as 5α-reductase inhibitors. Thirdly, the structural characterization of the novel receptors located in the cell membranes would help in understanding the molecular mechanisms of action and in turn provide a basis in designing 5αPR binding antagonists and 3αHPR agonists. Fourthly, more information is needed on the mechanisms of action and the involved cell signaling pathways, particularly for 3αHP. Fifthly, with respect to their metastatic potential, effects of the P metabolites on angiogenesis and cell-to-cell as well as cell-to-matrix interaction molecules need to be explored. Sixthly, the identification of factors that alter expression of 5α-reductase and HSOs, resulting in changes in 5α-pregnane:4-pregnenolone ratios, may give insight into processes that initiate deregulation of P metabolite balance.

In addition to raising the status of the P metabolites from waste products to active hormones and to providing an alternative endocrine-based hypothesis for human breast cancer, the findings suggest new biomarkers, diagnostic tests, and therapeutic regimens that may be applicable to both estrogen-sensitive and -insensitive normal and cancerous human breast tissues (Wiebe et al. 2005). Biomarkers and diagnostic tests might be based on measurements of P metabolite concentrations in nipple aspirates, changes in 5α-reductase and HSO activities and gene expression, and/or 5αP receptor concentrations in biopsies. Therapeutic regimens might involve (a) actively decreasing 5αP and increasing 3αHP by blocking 5α-reductase and stimulating 3α-HSO activities and gene expression, (b) blocking the binding of 5αP to its receptor, and (c) down-regulating 5αP receptor and up-regulating 3αHP receptor levels.

In light of the findings regarding the P metabolites in relation to breast cancer, it appears pertinent to stress the importance of the intra- and para-cellular metabolomic microenvironments generated by the cells and potentially responsible either for maintaining normalcy or for transition/progression to neoplasia. It would seem propitious to consider therapies for breast cancer to be applied directly to the affected tissues via local depots or targeted infusions rather than the whole-body-every-tissue mode of current ingestion routes. Thus, it is hoped that the evidence presented in this review will stimulate further research into the potential roles of P metabolite hormones in breast cancer and generate new ideas for its control, regression, and prevention.

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