Mesenchymal-epithelial interactions and breast cancer – role of local estrogen production

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Introduction

The three key stages of breast cancer are initiation, growth and development, and metastasis. The initiating events in breast cancer are genetic and may be either familial or sporadic in origin, the former arising as a consequence of congenital mutations and the latter as a consequence of changes stemming from environmental insults. Whereas the identification of the BRCA1 and 2 genes is a promising start, this work must be considered only the beginning of the search for genetic mutations in breast cancer. Similarly, whereas mutations in suppressor genes such as p53 and RB, or overexpression of oncogenes such as c-erbB2 and c-myc occur relatively frequently in breast cancer, as yet few strategies have been developed to exploit this knowledge, nor has any mutation been described which comes close to being a universal marker of any breast cancer subtype. Following initiation, breast cancer development is driven by hormonal and other locally produced factors such as cytokines. The reason that women are at much higher risk than men of developing breast cancer is that women produce estrogens in abundance from the time of puberty, whereas men do not. Metastasis is the main cause of death from breast cancer and bone is a favored site. One reason may again be local production of factors in bone cells which facilitate either tumor invasion or growth, such as cytokines and estrogens. In consideration of both the growth and development of the primary tumor as well as its subsequent metastasis, it is clear that a major factor involved is estrogen, yet the incidence of breast cancer increases with advancing age in spite of the fact that the ovaries cease to produce estrogens after the time of menopause. So an important question arises as to the source of the estrogen driving breast cancer development in elderly women.

Sources of estrogen in elderly women

Plasma levels of estrogen decrease markedly at the time of menopause due to the cessation of ovarian secretion. Nevertheless, it is apparent that other sources of estrogen are available to postmenopausal women. In the 1970s, MacDonald, Siiteri, and their colleagues studied the extragonadal production of estrogens in human volunteers by infusing $[^3]$H]androstenedione into their blood and measuring the fractional conversion to estrone (Hemsell et al. 1974, Edman & MacDonald 1978, MacDonald et al. 1978). It was found that in normal weight individuals about 1% of the androstenedione was converted to estrone; however, strikingly, in obese individuals, this could increase as much as tenfold. A similar pattern was found in both men and women. These results suggest strongly that most of the extragonadal
conversion of circulating androstenedione to estrone occurs in the adipose tissue.

Of equal interest was the finding that when the data were transformed so that the fractional conversion of circulating androstenedione to estrone was plotted as a function of the age of the individuals, there was also a striking increase with advancing age, suggesting that the capacity of adipose tissue to synthesize estrogens increases with age (Fig. 1). Further support for this concept was derived from studies performed several years later in which stromal cells prepared from human adipose tissue derived from women of various ages were examined for their capacity to convert androstenedione to estrone, and it was found that indeed cells from elderly individuals had a higher capacity for estrone synthesis than those derived from young individuals (Cleland et al. 1985). This increase in the capacity of adipose tissue to synthesize estrogens from circulating precursors correlates well with the incidence of endometrial cancer, which is essentially a disease of elderly obese women (Edman & MacDonald 1976). However, as mentioned above, the incidence of breast cancer also increases with advancing age, and also is higher in obese individuals. Further support for the role of estrogen synthesized in adipose tissue as an important player in the incidence of breast cancer in the elderly comes from a consideration of the efficacy of surgical ablation in the treatment of breast cancer, namely oophorectomy and adrenalectomy. While it is readily apparent why oophorectomy should be efficacious in

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\begin{align*}
C_{19} \text{Steroids} & \quad + \quad 3 \text{O}_2 \\
& \quad \xrightarrow{\text{Reductase}} \quad P450\text{arom} \\
C_{18} \text{Steroids} & \quad + \quad 3 \text{H}_2\text{O}
\end{align*}
\]

Figure 2 The aromatase enzyme complex as it occurs in the endoplasmic reticulum of cells in which it is expressed. The aromatase reaction is catalyzed by P450arom, a member of the cytochrome P450 superfamily. Associated with it is a flavoprotein, NADPH-cytochrome P450 reductase, which is an essentially ubiquitous protein in the endoplasmic reticulum of most cell types, and is responsible for transferring reducing equivalents from NADPH to any microsomal form of cytochrome P450 with which it comes into contact.
In this context in younger women, it is less clear why adrenalectomy would be a useful therapeutic procedure considering that the human adrenal cortex does not synthesize estrogens. However, when one considers that the principal source of substrate utilized by adipose tissue to synthesize estrogens is circulating androstenedione produced by the adrenal cortex, then the rationale for this approach becomes clear.

In spite of the evidence implicating estrogens in the development of breast cancer in the elderly, it has proven impossible to establish a correlation between plasma levels of estrogen and the incidence of breast cancer (England et al. 1974, McFayden et al. 1976, Reed et al. 1983). These results suggest that the source of the estrogen which is the major player may be local production within the breast itself. In contrast to the low levels of estrogens found in plasma, a number of investigators have examined the levels of estrogens in normal and in malignant breast tissues (Bonney et al. 1983, Van Landeghem et al. 1985, Vermeulin et al. 1986). From these studies, it is apparent that concentrations of both estrone and estradiol are significantly higher in both normal and malignant breast tissue than the levels found in plasma. Furthermore, estrogen levels are higher in malignant than in normal breast tissue, and estradiol levels are higher than those of estrone in malignant tissue. In most of these studies, the levels of estradiol and estrone were between 10- and 20-fold of those found in plasma based on units of ng/g wet weight in the case of the solid tissue and ng/ml in terms of the plasma. These elevated estrogen levels in breast tissue could arise by either of two mechanisms: increased uptake from the plasma or increased local synthesis. If the former is the case then the high levels of estrogen found in breast tumors should correlate with the levels of estrogen receptors. No such correlation has been found, suggesting that the reason that estrogen levels are higher in malignant and in normal breast tissue as compared with plasma is because of local synthesis within the breast adipose tissue and within the breast tumor (Fishman et al. 1977, Abul-Hajj 1979, Edery et al. 1981).

In an attempt to address this issue, Reed et al. (1989) employed a double isotope infusion technique to distinguish uptake of estrogen from the circulation from in situ estrogen synthesis in normal and malignant breast tissues. They found that in some malignant breast tissues up to 90% of the estrone content of the tumors was identified as resulting from in situ formation. In another important study, O'Neal & Miller (1987) and O'Neal et al. (1988) measured aromatase activity in adipose tissue from breast quadrants removed at the time of mastectomy for the presence of a tumor. They found that, almost without exception, aromatase activity was highest in adipose derived from the quadrant that contained the tumor as compared with the other quadrants in the breast, suggesting that cross-talk existed between the tumor and the surrounding adipose tissue in terms of the ability of the latter to synthesize estrogens. This result was subsequently confirmed by another group (Reed et al. 1993, Purohit et al. 1995), however, a third study yielded conflicting results (Thijssen et al. 1991). Nevertheless, taken together, these studies suggested that local estrogen production within the breast is related to the presence of a tumor and that further studies of this phenomenon were warranted.

The aromatase enzyme and its gene

The biosynthesis of estrogens is catalyzed by a microsomal member of the superfamily of enzymes known collectively as cytochrome P450, namely aromatase cytochrome P450 (P450arom, the product of the CYP19 gene) (Thompson & Sitiery 1974, Mendelson et al. 1985, Nakajin et al. 1986, Kellis & Vickery 1987, Osawa et al. 1987, Nelson et al. 1993) (Fig. 2). This heme protein is responsible for binding the C19 steroid substrate and catalyzing the series of reactions leading to formation of the phenolic A ring characteristic of estrogens. Associated with the P450arom is a flavoprotein, NADPH-cytochrome P450 reductase (Simmons et al. 1985), which is an essentially ubiquitous protein in the endoplasmic reticulum of most cell types, and is responsible for transferring reducing equivalents from NADPH to any microsomal form of cytochrome P450 with which it comes into contact. Whereas the reductase is the product of a single gene, cytochrome P450arom is a member of a flourishing superfamily of genes, namely the cytochrome P450 family, which contains at the present time over 300 characterized members belonging to 36 gene families (Nelson et al. 1993). Within this, cytochrome P450arom is presently the sole member of gene family 19, designated CYP19. This designation is based on the
fact that the C19 angular methyl group is the site of attack by oxygen.

Some years ago we and others cloned and characterized the CYP19 gene which encodes human P450arom (Means et al. 1989, Harada et al. 1990, Toda et al. 1990) (Fig. 3). The coding region spans 9 exons beginning with exon II. Sequencing of RACE-generated cDNA clones derived from P450arom transcripts present in the various tissue sites of expression revealed that the 5'-termini of these transcripts differ from one another in a tissuespecific fashion upstream of a common site in the 5'-untranslated region (Means et al. 1991, Kilgore et al. 1992, Jenkins et al. 1993, Toda & Shizuta 1993). Using these sequences as probes to screen genomic libraries, it was found that these 5'-termini correspond to untranslated exons which are spliced into the P450arom transcripts in a tissue-specific fashion, due to the use of tissue-specific promoters. Placental transcripts contain at their 5'-ends untranslated exon I.1 which is located at least 40 kb upstream from the start of translation in exon II (Mahendroo et al. 1991, Means et al. 1991). This is because placental expression is driven from a powerful distal placental promoter, I.1, upstream of untranslated exon I.1. On the other hand, transcripts in the ovary contain sequence at their 5'-ends which is immediately upstream of the start of translation. This is because expression of the gene in the ovary utilizes a proximal promoter, promoter II (Jenkins et al. 1993). By contrast, transcripts in adipose tissue contain yet another distal untranslated exon, I.4, which is located in the gene 20 kb downstream from exon I.1 (Mahendroo et al. 1993). Additionally, adipose tissue contains transcripts specific for promoter II, as well as those containing exon I.3, which contain promoter II as part of the exonic sequence, and share common regulatory elements with promoter II. A number of other untranslated exons have been characterized by ourselves and others (Harada et al. 1993, Toda et al. 1994), including one specific for brain (Honda et al. 1994). Splicing of these untranslated exons to form the mature transcripts occurs at a common 3'-splice junction which is upstream of the start of translation. This means that although transcripts in different tissues have different 5'-termini, the protein encoded by these transcripts is always the same regardless of the tissue site of expression, thus there is only one human P450arom enzyme encoded by a single-copy gene.
Expression of aromatase in adipose tissue

We have found that aromatase expression in adipose tissue does not occur in adipocytes but rather in the stromal cells which surround the adipocytes, and which may themselves be preadipocytes (Price et al. 1992). These stromal cells grow in culture as fibroblasts. Consequently we have employed these cells in primary culture as a model system to study the regulation of estrogen biosynthesis in adipose tissue (Ackerman et al. 1981). When serum is present in the culture medium, expression is stimulated by glucocorticoids including dexamethasone (Simpson et al. 1981). Under these conditions P450arom transcripts contain primarily untranslanted exon I.4 at their 5'-ends (Mahendroo et al. 1993, Zhao et al. 1995a). We have subsequently characterized the region of the CYP19 gene upstream of exon I.4 (Fig. 3) and found it to contain a TATA-less promoter, as well as an upstream GRE and an Sp1 sequence within the untranslated exon, both of which are required for expression of reporter gene constructs in the presence of serum and glucocorticoids (Zhao et al. 1995a). Additionally, we found this region to contain a GAS (interferon-γ activating sequence) element. Such sequences are known to bind transcription factors of the signal transducers and activators of transcription (STAT) family (Schindler et al. 1992, Darnell et al. 1994, Zhong et al. 1994). On the other hand, when the cells are maintained in serum-free medium, expression is stimulated by cyclic AMP (cAMP), and this action is markedly potentiated by phorbol esters, although these have no action by themselves. Under these circumstances P450arom transcripts contain primarily promoter II and I.3-specific sequences at their 5'-end. Analysis of the region of the CYP19 gene upstream of promoter II reveals that it contains a binding site for the orphan member of the steroid hormone receptor family, steroidalogenic factor I (SF-1). SF-1 is a critical developmental factor for the gonads as well as the adrenals. Upstream of the SF-1 site is a sequence TGCACGTCA, identical to a canonical cAMP response element (CRE) save for the extra 'C'; and hence known as a CRE-like element (CLS). This element proved to be a weak binding site for the transcription factor CREB (Michael et al. 1997). Both of these sites were shown to be essential for basal and cAMP-stimulated expression of reporter gene constructs in primary cultures of ovarian granulosa and corpus luteum cells.

Recently we observed (Zhao et al. 1995b) that the effect of serum to stimulate aromatase expression in human adipose stromal cells (in the presence of glucocorticoids) can be mimicked by specific factors, namely members of the class I cytokine family, which includes interleukin-11 (IL-11), IL-6, oncostatin-M (OSM), and leukemia inhibitory factor (LIF) (Narazaki et al. 1994, Stahl et al. 1994). Members of this cytokine family employ a receptor system involving two different Jak associated components, gp130 and a related β-component (LIFRβ) (Stahl & Yancopoulos 1993). However, the IL-6 receptor complex includes a component whose cytoplasmic domain is apparently not involved in signalling (Kishimoto et al. 1992), and which can exist in a soluble form (Kishimoto et al. 1992). Stimulation by IL-6 required addition of the IL-6 soluble receptor to the culture medium. Recently an α-subunit of the IL-11 receptor complex has been cloned (Hilton et al. 1994), although this does not apparently exist in a soluble form. The concentration dependence of the stimulation of aromatase by IL-6, IL-11, LIF, and OSM is indicative of high-affinity receptor binding. Using fibroblasts derived from breast tumor tissue, Reed and colleagues also showed that IL-6 could stimulate aromatase activity (McDiarmid et al. 1994), and that this action was potentiated by addition of the IL-6 soluble receptor (Singh et al. 1995).

Addition of class I cytokines to adipose stromal cells resulted in a rapid phosphorylation of Jak1 kinase (Zhao et al. 1995b). By contrast, Jak3 kinase was not phosphorylated under these conditions to any significant extent, whereas Jak2 kinase was phosphorylated to an equal extent both in the presence or absence of IL-11. As indicated by blotting with an anti-phosphotyrosine antibody and by inhibition in the presence of herbimycin A, this phosphorylation occurred on tyrosine residues present in the Jak1 kinase. Both gp130 and LIFRβ can associate with and activate at least three members of the Jak family, Jak1, Jak2 and Tyk2, but utilize different combinations of these in different cells (Stahl et al. 1994); however, it is apparent that Jak1 is the kinase of choice in human adipose stromal cells.
This action results in the rapid phosphorylation of STAT3 on tyrosine residues, but this was not the case for STAT1. Recently it has been shown that STAT3 is the substrate of choice for the IL-6/LIF/OSM cytokine receptor family, and that the specificity of STAT phosphorylation is based not upon which Jak kinase is activated (Boulton et al. 1994, Stahl et al. 1994, Zhong et al. 1994), but rather is determined by specific tyrosine-based motifs in the receptor components, namely gp130 and LIFRβ, shared by these cytokines (Stahl et al. 1995). Finally, gel shift analysis indicated that STAT3 can interact with the GAS element present in the promoter I.4 region of the P450arom gene upon addition of IL-11 to these cells. This interaction in turn results in activation of expression, as indicated by transfection experiments employing chimeric constructs in which the region -330/+170 bp of the I.4 promoter region was fused upstream of the CAT reporter gene. The results indicate that both deletion of the GAS sequence, as well as mutagenesis of this sequence, resulted in complete loss of IL-11- and serum-stimulated expression in the presence of glucocorticoids.

Activation of this pathway of expression by these cytokines is absolutely dependent on the presence of
glucocorticoids. This action of glucocorticoids is mediated by the GRE element downstream of the GAS element (Zhao et al. 1995a). Additionally, the Sp1-like element present within untranslated exon I.4 is also required, at least for expression of the -330/+170 bp construct (Zhao et al. 1995a). These sequences, while present within a 400 bp region of the gene, are not contiguous and the nature of the interaction between STAT3, the glucocorticoid receptor and Sp1 to regulate expression of the P450arom gene via the distal promoter I.4 remains to be determined. Our present understanding of the regulation of expression of aromatase in adipose tissue by class I cytokines is summarized in Fig. 4.

Recently, we have found that tumor necrosis factor α (TNFα) also stimulates aromatase expression in adipose stromal cells in the presence of dexamethasone. This action of TNFα is mimicked by ceramide, indicative that sphingomyelinase activity may be involved in the TNFα response. This action of TNFα appears to involve promoter I.4, specifically an AP1 site upstream of the GAS element (Fig. 3) which binds a c-jun/fos heterodimer upon activation by TNFα (Zhao et al. 1996a). Reed and colleagues observed a similar stimulatory action of TNFα employing tumor-derived fibroblasts (McDermid et al. 1994).

We also developed a competitive RT-PCR technique to measure the levels of the various P450arom transcripts in adipose tissue in different body sites of women of various ages. We found a marked increase in total aromatase expression in abdomen, buttocks and thighs with increasing age, thus providing a molecular basis for the previously described in vivo data (Hemsell et al. 1974, Edman & MacDonald 1978, MacDonald et al. 1978). We also found that in the abdomen, buttocks and thighs of these subjects, I.4-containing transcripts predominated, with I.3- and II-specific transcripts in much lower abundance (Agarwal et al. 1996a). This information provides clues as to the factors responsible for regulating aromatase expression. Based on these findings we suggest that aromatase expression in adipose tissue may be under tonic control by circulating glucocorticoids and that regional and age-dependent variations may be the consequence of paracrine and autocrine secretion of stimulatory cytokines such as IL-6 and TNFα, the levels of which have been shown to increase with age (Wei et al. 1992, Daynes et al. 1993).

Expression of aromatase in breast tissue

As indicated previously, a number of earlier studies have shown that the levels of estrogens and the activity of aromatase are high in regions of the breast proximal to a tumor or else within the tumor itself. With the analysis of the structure of the aromatase CYP19 gene, it became apparent that a study of the regional distribution of aromatase expression within the breast was warranted. Because of the low levels of aromatase transcripts in adipose tissue, it was necessary to perform such a study by means of competitive RT-PCR. In three separate studies, such a correlation has now been obtained supporting the earlier studies on the distribution of aromatase activity (Bulun et al. 1993, Harada et al. 1993, Soudain et al. 1996) (Fig. 5). In one of these studies, a comparison was made between CYP19 gene expression and aromatase activity in breast cancers (Soudain et al. 1996). Although such a correlation was observed, it did not reach statistical significance because in a minority of tumors aromatase activity was low, although transcript level was high.

**Figure 5** Amplification of coding region (total transcripts) of CYP19 gene transcripts in cDNA from 1 µg RNA isolated from different breast quadrants of cancer patients and cancer-free individuals. Data are presented as mean±S.E.M. and are normalized to GAPDH expression. Q1 and Q2-Q4 are the different quadrants of cancer patients. T, tumor (n=9); Q1, tumor-bearing quadrant (n=15); Q2-Q4, average of non-tumor-bearing quadrants Q2, Q3, and Q4 (n=15); CT, average of four quadrants of three cancer patients with a centrally located tumor; CF, average of three regions from both breasts of nine cancer-free patients. Reproduced from Agarwal et al. (1996b) with permission.
suggesting the presence of an endogenous inhibitor of activity. In another of these studies, an attempt was made to correlate aromatase expression with the ratio of adipocytes to stromal cells within the adipose tissue employing quantitative morphometric methods (Bulun et al. 1993). In disease-free breast tissue of premenopausal women, it was found that two-thirds of the highest fibroblast to adipocyte ratios and of the highest P450arom transcripts levels were detected in an outer breast region whereas in only one patient was the highest value detected in an inner region (Bulun et al. 1996). This distribution pattern directly correlates with the most common or the least common sites of carcinoma of the breast, the outer and inner regions respectively. Moreover, a direct relationship was demonstrated between ratio of adipose fibroblasts and P450arom transcripts within the breast, such that regions with the highest fibroblast to adipocyte ratios contained the highest P450arom transcript levels. These results suggest that adipose tissue aromatase expression in the disease-free breast is determined by the local ratio of fibroblasts to adipocytes. When a similar study was conducted employing tissue taken from a breast bearing a tumor, a similar result was obtained (Bulun et al. 1993). Based on these results, it is possible to propose the following model. Although the events which initiate breast tumor formation are uncertain, they are genetic in nature, either sporadic or familial. Once tumor formation has initiated, the rate of development of the tumor will depend on the ratio of fibroblasts to adipocytes in the region of the breast in which the tumor is located, and thus the local concentration of estrogen to which it is exposed.

However, this is clearly not the whole story since it is now apparent that breast tumors produce factors which influence the local aromatase expression in the surrounding tissue and indeed in the tumor itself. Evidence that this was the case was first obtained by Reed et al. (1993) employing fibroblasts derived from breast tumors or from breast adipose tissue. Using these cells, breast cystic fluid, human serum albumin, and conditioned medium from tumor-derived fibroblasts were all found to markedly stimulate aromatase activity, but only in the presence of dexamethasone. Subsequently, Nichols et al. (1995) found that a heat-labile factor was present in condition medium from T47D breast cancer cells which also stimulated aromatase activity of adipose stromal cells in the presence of dexamethasone.

Based on the work previously discussed that class I cytokines and TNFα stimulate aromatase expression of adipose stromal cells or breast-derived stromal cells in the presence of dexamethasone, these results suggest that one or more of these cytokines might be mediating this stimulatory effect. Consistent with this, breast cystic fluid has been found to contain high concentrations of IL-6, as well as IL-1, IL-2, IL-8, and OSM (Reed et al. 1992, Lai et al. 1994). Conditioned medium from MDA-MB-231 breast cancer cells was also found to contain a stimulatory factor which eluted on ion exchange chromatography at a concentration of sodium chloride similar to that used to elute IL-6. Furthermore, Crichton et al. (1996) using RT-PCR found that a number of class I cytokines were expressed in every breast tumor sample tested and that, moreover, T47D cells expressed IL-11. Adipose stromal cells themselves expressed IL-6, IL-11, LIF, but not OSM nor the IL-6 receptor. These observations, however, do not provide an explanation for the stimulatory effect of serum albumin, which will be discussed later.

Taken together with the observation that aromatase expression in breast tissue is higher in the tumor and in the quadrant containing the tumor than in the other quadrants of the tumor-containing breast, and that expression in these quadrants in turn is higher than expression in healthy breast tissue (Agarwal et al. 1996b), these results strongly suggest that the tumor produces a factor or factors which stimulate local aromatase expression in a fashion resembling a concentration gradient of stimulatory factor emanating from the tumor. Furthermore, given the presence of cytokines known to stimulate stromal cell aromatase expression in breast tumors, breast cancer cells and breast cystic fluid, as well as in adipose tissue, together with the fact that in samples of adipose tissue, promoter 1.4-containing transcripts of aromatase are predominant, these results add credence to the view that one or more cytokines of the class I category or else TNFα mediate this response.

These factors could be produced either by the tumorous epithelium itself, the surrounding adipose tissue, or else from cells of the immune system which infiltrate the tumor site. A dramatic example of this was provided by a study of Reed and colleagues of a normal woman undergoing reduction mammoplasty who had previously had breast augmentation by
silicone injection not contained within a capsule (Purohit et al. 1995). In tissue from this patient, there was evidence of chronic inflammation and a marked macrophage response. Aromatase activity of breast tissue from this patient was considerably higher than that detected in mastectomy adipose tissue samples, and a significant correlation was found between aromatase activity and IL-6 production by tissue explants.

In order to gain insight into the factors which stimulate aromatase expression in the breast tissue of individuals with breast cancer, a number of studies have examined the distribution of the various 5'-termini-specific aromatase transcripts in samples of breast adipose tissue removed from cancer-free individuals as well as individuals diagnosed as having breast cancer (Agarwal et al. 1996b, Harada et al. 1996, Zhou et al. 1996). In breast adipose tissue samples from cancer-free individuals, it has been found that promoter I.4-specific transcripts were the most numerous, comprising at least 50% of the total P450arom transcripts, whereas promoter I.3- and promoter II-specific transcripts together made up the remaining 50% (Agarwal et al. 1996a). This is consistent with aromatase expression in adipose tissue of cancer-free individuals being regulated mainly by class I cytokines or else TNFα, as previously discussed. Thus in normal human adipose tissue it appears that aromatase expression is under local control by such factors via paracrine and autocrine mechanisms.

Of considerable surprise therefore was the observation that the increase in total P450arom expression that occurs in breast adipose tissue of patients with cancer was accompanied by only a small increase in promoter I.4-driven expression, but rather was largely the result of an increase in expression from promoter II and promoter I.3 (Agarwal et al. 1996b, Harada et al. 1996, Zhou et al. 1996). This was true not only in the quadrant which

**Figure 6** Amplification of exon-specific CYP19 gene transcripts in cDNA from 1 μg RNA isolated from different breast quadrants of cancer patients and cancer-free individuals. Data are presented as mean ± S.E.M. of percent of total transcripts (i.e. coding region) and are normalized to GAPDH expression. PI, promoter I-specific transcripts; I.3, exon I.3-specific transcripts; I.4, exon I.4-specific transcripts. Percentage was calculated considering coding region as 100. Other symbols and abbreviations are discussed in the legend to Fig. 5. Reproduced from Agarwal et al. (1996b) with permission.
Figure 7 Schematic representation of regulation of aromatase gene expression in human adipose stromal cells by PGE$_2$. PGE$_2$ binds to EP2 receptors on the cell surface resulting in activation of adenylyl cyclase and increased cAMP formation. As a consequence, protein kinase A (PK-A) is activated and phosphorylates CREB. CREB binds weakly to a CLS upstream of promoter II and, together with SF-1, activates transcription of aromatase (Michael et al. 1997). Other details of the mechanism such as whether or not SF-1 is activated, and the role of the protein kinase C pathway in potentiation of the cAMP signal, remain to be resolved.

contained the tumor but in the other breast quadrants as well. This conclusion is illustrated diagrammatically in Fig. 6. These results imply that, in the presence of a tumor, factors which stimulate aromatase expression via promoter I.4 are not primarily responsible for the increase in expression in surrounding adipose fibroblasts, but rather some other influence stimulating expression via promoter II and I.3 is dominant. As indicated previously, expression from promoter II and I.3 is stimulated by dibutyril cAMP (Mendelson et al. 1986, Mahendroo et al. 1993). Moreover this action of cAMP analogs is dramatically potentiated by phorbol esters. Thus it would appear that in the presence of a tumor, P450arom expression is driven primarily by a factor or factors which stimulate adenylyl cyclase, perhaps in conjunction with protein kinase C activation. Such a limitation rules out many potential candidates, including most cytokines.

A recent study suggests that the factor in question is prostaglandin (PG)E$_2$ (Zhao et al. 1996b). In this report, it was shown that PGE$_2$ is the most potent factor which stimulates aromatase expression via cyclic AMP and promoter II. PGE$_2$ acts via EP1 and EP2 receptor subtypes to stimulate both the protein kinase C and protein kinase A pathways respectively. The combined stimulation of both of these pathways results in maximal expression of promoter II-specific CYP19 transcripts. The pathway whereby signalling via the EP2 receptor and cAMP may act to stimulate aromatase expression is illustrated in Fig. 7. PGE$_2$ has been reported to be present at high levels in malignant human breast tumors and is synthesized by several human breast cancer cell lines (Rolland et al. 1980, Schrey & Patel 1995). It is also synthesized by tumor fibroblasts (Schrey & Patel 1995) and adipose stromal cells (Mitchell et al. 1983) as well as by macrophages (Yamamoto et al. 1995) which have been shown to infiltrate many breast tumor sites (Purohit et al. 1995). Thus, breast tumors provide a potentially rich source of PGE$_2$ which can stimulate aromatase expression both in the tumor itself and in the surrounding adipose tissue. The resulting increased estrogen biosynthesis in local sites in turn may result in increased growth and development of the tumor. These observations are also consistent with the previously noted observations that serum albumin is stimulatory of aromatase expression in breast fibroblasts. Serum albumin contains bound fatty acids which can be readily taken up by the cells.
and converted to prostaglandins. It was previously shown that adipose stromal cells synthesize prostaglandins at a brisk rate when incubated with serum, which contains albumin (Mitchell et al. 1983).

These considerations are also consistent with recent evidence which suggests that polyunsaturated fatty acids stimulate the growth and development of mammary tumors (Carroll 1981), and with epidemiological evidence from a recent study which examined the association of non-steroidal anti-inflammatory drugs and breast cancer risk in a case-controlled study of 511 breast cancer patients and 1534 population control subjects (Harris et al. 1996). The results indicated that the non-steroidal anti-inflammatory drugs may have chemo-preventive potential against the development of breast cancer. The study described here points to at least one mechanism whereby this could occur, and suggests that PGE2 produced by breast tumors or by infiltrating macrophages can indirectly increase the growth and development of breast tumors, namely by stimulating the production of estrogen in local sites within the breast as a consequence of epithelial-mesenchymal interactions.

**Mesenchymal-epithelial interactions in regulation of aromatase expression in breast tissue**

Throughout this discussion, emphasis has been placed on the fact that, in adipose tissue, aromatase expression occurs in the stromal cells rather than the adipocytes themselves. Aromatase expression also occurs in fibroblasts derived from breast tumors. An issue has arisen as to whether aromatase expression also occurs in epithelial tissue of the breast, either normal or malignant. Immunocytochemical studies by two laboratories have indicated that aromatase expression occurs exclusively or predominantly in mesenchymal cells and that epithelial cells have little or no detectable expression (Sasano et al. 1994, Santner et al. 1997). However, in one study aromatase expression was detected in epithelial cells at least to the same degree as in mesenchymal cells (Lu et al. 1996). These studies used different methodologies for fixing and staining of the tissue, as well as different anti-aromatase antibodies, and clearly this issue needs to be resolved.

Regardless of whether aromatase expression occurs in epithelial tissue, it certainly occurs in the mesenchyme. An important issue which arises in this context is the nature of the C18 product produced as a consequence of mesenchymal aromatase expression. It has already been indicated that in postmenopausal women the major substrate utilized by adipose tissue aromatase is circulating androstenedione produced by the adrenal cortex. As a consequence of this, the initial product of aromatase activity is not estradiol, but rather estrone which is essentially inactive as an estrogen. In spite of this, as indicated previously, estradiol levels are high in breast tumor samples and, indeed, the levels are higher than those of estrone. These considerations point to the presence of 17β-hydroxysteroid dehydrogenase (17β-HSD) in breast tissue. The isoform of 17β-HSD which is most effective in converting estrone to estradiol is 17β-HSD type I. Several studies have indicated that this enzyme is present, not in mesenchymal cells, but specifically in breast carcinoma cells. For example, in a recent immunocytochemical study by Sasano et al. (1996), it was found that marked aromatase immunoradioactivity was observed in stromal cells around carcinomatous glands in 78% of cases, whereas 17β-HSD type I immunoradioactivity was detected in the carcinoma cells in 56% of cases. Aromatase and 17β-HSD were not always expressed simultaneously in the human breast carcinoma, but their simultaneous expression was more frequent in invasive lobular carcinoma than in invasive ductile carcinoma. Earlier studies were indicative of the presence of 17β-HSD type I in cultured breast cancer cells such as MCF7 cells (Duncan et al. 1994). Moreover, in these cells 17β-HSD activity was increased by IL-6 and TNFα, factors which also stimulate aromatase activity in mesenchymal cells as discussed above. Thus it appears that estrone produced in the mesenchymal cells of the breast can be efficiently converted to estradiol in breast carcinoma cells themselves.

Based on these various considerations, it is possible to propose a regulatory feedback (Fig. 8) loop whereby tumorous breast epithelium or else macrophages and lymphocytes recruited to the tumor site produce factors such as PGE2, TNFα and class I
cytokines which stimulate the surrounding mesenchymal cells both within the tumor and in the adipose tissue to express aromatase, and the carcinoma cells themselves to express 17β-HSD type I. The resulting locally produced estradiol acts to stimulate the growth and development of the tumor either directly or by stimulating the production of growth factors. Estradiol might also stimulate the production by the tumor of factors which stimulate aromatase expression such as PGE₂ or cytokines, since it has been shown that estradiol can stimulate IL-11 production in T47D cells (Crichton et al. 1996). Thus it is possible to envision the establishment of a positive feedback loop leading to the ever-increasing growth and development of the tumor via paracrine and autocrine effects mediated by mesenchymal-epithelial interactions. Since aromatase expression in adipose tissue occurs exclusively in the undifferentiated fibroblasts, we consider aromatase expression to be a marker of this undifferentiated phenotype. Consistent with this, factors which stimulate aromatase expression in these fibroblasts such as class I cytokines and TNFα are known to inhibit adipocyte differentiation in a cell culture model, namely the 3T3L1 cell line. Indeed, TNFα can reverse the lipid-laden adipocyte phenotype. Prostaglandins also stimulate lipolysis by adipocytes via an increase in the activity of hormone-sensitive lipase due to stimulation of adenylate cyclase.

**Perspectives**

The considerations presented here open the possibility of the development of inhibitors of aromatase expression in adipose tissue which may be of utility in the management of breast cancer. Currently, a new generation of aromatase inhibitors is being introduced for the clinical management of breast cancer as alternatives to estrogen antagonists. These compounds have high specificity and affinity for aromatase but, as indicated previously, the aromatase protein is the same regardless of the tissue site of expression. Consequently these compounds inhibit aromatase in all tissue sites of expression. In the case of postmenopausal women, this could be contraindicated; for example, local estrogen production in bone may be important in the prevention of osteoporosis, and local estrogen production in the brain may be important in the maintenance of cognitive function and prevention of Alzheimer’s disease. Consequently factors which inhibit, not aromatase activity, but rather aromatase
expression uniquely in adipose tissue, could be of utility in the treatment and management of breast cancer in postmenopausal women. The possibility that prostaglandin inhibitors might play such a role is worthy of serious consideration in this context. Furthermore, since aromatase is a marker of the undifferentiated adipocyte fibroblast phenotype, factors which stimulate the differentiation of adipocytes might also prove to be inhibitory of aromatase expression in adipose and thus could also find utility in breast cancer management, and should also be considered seriously in this contest.

Acknowledgements

This work was supported, in part, by USPHS grants no. R37-AG08174 (to E R S) and no. CA67167 (to S E B), and by US Army Award no. DAMD17-97-J-4188 (to S E B). V R A was supported, in part, by USPHS Training Grant no. 5-T32-HD07190. The authors gratefully acknowledge the skilled editorial assistance of Susan Hepner.

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