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Aromatase Gene Expression in Adipose Tissue: Relationship to Breast Cancer

Serdar E. Bulun,* Mala S. Mahendroo and Evan R. Simpson

Cecil H. and Ida Green Center for Reproductive Biology Sciences and the Departments of Obstetrics/Gynecology and Biochemistry, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75235, U.S.A.

Recent studies have established that concentration gradients of aromatase expression occur within the breast, with the highest levels of expression occurring in sites proximal to a tumor. These variations in aromatase expression correlate with regional differences in the relative proportions of the histologic components of breast adipose tissue, in particular adipocytes and stromal cells, since regions containing the highest numbers of stromal cells are the sites of elevated aromatase transcript levels. Although the initiating events are unknown, it is proposed that, once neoplastic cells start to replicate, tumor growth will be promoted by locally increased estrogen levels. In turn, growth factors produced by the tumor in response to locally increased estrogen levels may further increase aromatase expression in the surrounding adipose tissue. Thus a positive feed-back loop is established in which locally-produced estrogens and tumor-derived growth factors act by paracrine and autocrine mechanisms to sustain the growth and development of the tumor. Further support for this concept is obtained from the observation that aromatase expression in breast adipose is regulated by enhancer elements that appear to respond positively to growth factors, in contrast to expression in granulosa cells, which is inhibited by growth factors.

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INTRODUCTION

Formation of estrogens from C_{19} steroids is catalyzed by a specific microsomal form of cytochrome *P*450, aromatase cytochrome *P*450 (*P*450arom, the product of CYP19 gene). A flavoprotein, NADPH–cytochrome *P*450 reductase, which is a ubiquitous component of the endoplasmic reticulum of most cells [1, 2], transfers reducing equivalents from NADPH to the *P*450arom. Aromatase expression occurs in a number of human tissues and cell types, including syncytiotrophoblast of placenta, [3], hydatid moles [4], JEG-3 cells (a choriocarcinoma-derived cell line) [5], fetal hepatocytes [6], ovarian granulosa cells [7], and testicular Leydig cells [8]. Additionally, in various species, the activity was shown to be present in Sertoli [9], Leydig [10, 11] and germ cells [12] in the male, and in several sites in the brain of both sexes [13]. In women, aromatase activity and *P*450arom transcripts have been detected both in breast adipose tissue and breast tumor tissue [14–20].

The principal site of estrogen formation in postmenopausal women is the adipose tissue [21, 22]. Estrogen production by adipose tissue increases as a function of age and obesity. The increased estrogen production in elderly obese women is believed to play a role in the pathogenesis of endometrial cancer. Furthermore, estrogen produced by adipose tissue within the breast may act locally to promote the growth of breast tumors [16, 23, 24]. The usefulness of estrogen antagonists as well as inhibitors of aromatase in the management of breast cancer has long been recognized. The implication of adipose tissue estrogen biosynthesis in the maintenance of growth of breast cancers is apparent from the palliative effects of adrenalectomy. Since estrogen production by adipose tissue is dependent for substrate on circulating androstenedione produced by the adrenal cortex, then the role of adrenalectomy is explicable in terms of the denial of substrate precursor for adipose tissue estrogen biosynthesis. It should be pointed out that the product of aromatase activity in adipose namely estrone, is a much weaker estrogen than estradiol. Evidence from at least two laboratories is indicative that 17β-hydroxysteroid dehydrogenase present in breast tumor tissue is capable of locally converting estrone into estradiol [25, 26].

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*Correspondence to S. E. Bulun.

Body fat distribution may be a contributing factor in the etiology of breast cancer. Some studies have indicated that individuals with central obesity have a greater risk of developing breast cancer [27]. However, Sellers *et al.* [28] demonstrated that the association of the waist-to-hip ratio with the risk of breast cancer was limited predominantly to women with a family history of breast cancer. Another study indicated that, in girls in early puberty, highest circulating estrogen levels were found in those with fat localized predominantly on the hips [29]. This is in agreement with our findings in that, compared with abdomen or thighs, the adipose tissue in the buttocks contains the highest levels of *P*450arom transcripts [30]. In addition, epidemiological studies are indicative of strong environmental factors in the incidence of breast cancer. Notably indicated are Western style diets with high fat content [31], although there is no conclusive evidence that fat consumption *per se* increases the risk of breast cancer.

Initially aromatase activity and subsequently, *P*450arom transcripts have been detected both in breast adipose as well as tumor tissue [14, 15, 17–20]. It has been proposed that the growth of breast carcinomas may be influenced by local estrogen biosynthesis in surrounding adipose tissue [16]. We previously demonstrated that aromatase activity in adipose stromal cells in culture is regulated primarily by changes in the levels of mRNA encoding *P*450arom [32]. More recently, we have investigated the expression of the CYP19 (*P*450arom) gene in breast adipose tissue as a function of proximity to a tumor and in breast cancer tissue *per se*, using an RT-PCR amplification procedure employing an internal standard to correct for variation in amplification from sample to sample.

REGULATION OF AROMATASE EXPRESSION IN HUMAN ADIPOSE

We have utilized human adipose stromal cells in culture as a model system to study the regulation of aromatase activity in human fat, having determined that aromatase activity and *P*450arom mRNA levels are much higher in the stromal elements of adipose tissue, *i.e.* the potential adipose precursor cells, than in adipocytes themselves [14, 17]. In fact, it is likely that the activity in the adipocyte fraction results from contamination by stromal elements. In these stromal cells, we have observed that aromatase expression is subject to complex and multifactorial regulation which is correlated with comparable changes in the levels of *P*450arom mRNA [32, 33]. In cultured human adipose stromal cells, aromatase activity is stimulated by glucocorticoids [34] and by cyclic AMP analogs [35]. The stimulatory effects of cyclic AMP are potentiated by phorbol esters [36] which activate protein kinase C, and are inhibited on the other hand by serum, as well as by a number of growth factors including EGF, TGFβ-1, TGFα, interleukin 1β, TNF, bFGF, and PDGF [36]. By contrast, the stimulatory action of glucocorticoids requires the presence of serum, but this action of serum can be mimicked in part by growth factors such as PDGF. The actions of these various stimulatory and inhibitory factors on enzyme activity are paralleled by comparable alterations in the levels of *P*450arom mRNA and in the synthesis of *P*450arom protein [32, 33]. By contrast, the NADPH–cytochrome *P*450 reductase component of the aromatase enzyme complex is much less markedly affected. On the other hand, in human ovarian granulosa cells in culture, aromatase activity and *P*450arom mRNA levels are elevated by FSH and cyclic AMP analogs [37]. However, whereas the FSH induction is antagonized by EGF, it is also antagonized by phorbol esters, in contrast to the situation with adipose stromal cells in culture. In addition, glucocorticoids have an inhibitory effect on aromatase expression in granulosa cells.

TISSUE-SPECIFIC REGULATION OF HUMAN CYP19 (*P*450arom) BY MEANS OF ALTERNATIVE PROMOTERS

In order to define the molecular mechanisms whereby stimulatory and inhibitory factors regulate tissue-specific expression of the *P*450arom gene in estrogen producing cells, as well as to characterize *trans*-acting factors and *cis*-acting elements required for tissue-specific and hormonal regulation of aromatase expression, we have isolated and characterized genomic clones that contain the entire human *P*450arom structural gene as well as flanking genomic DNA. The gene is greater than 75 kb in size, however the region encoding the *P*450arom protein spans about 35 kb of DNA and contains 9 exons (II–X) (Fig. 1). Exon II contains the translation initiation site. The gene has been mapped to chromosome 15 [38]. Recent findings from our laboratory are indicative that the human CYP19 gene contains a number of tissue-specific promoters which direct aromatase expression in human placenta, ovary and adipose tissue [39–41]. The majority of placental transcripts have 5′-termini containing sequences encoded in untranslated exon I.1 which lies at least 40 kb upstream of the translation start site (Fig. 1). In ovarian corpus luteum tissue, CYP19 gene transcription is initiated 120 bp upstream of the translation initiation site in exon II (promoter II). Some 20 kb downstream of exon I.1, another untranslated first exon, exon I.4, was found to be expressed in human breast adipose tissue [40]. Exon I.3, located 306 bp upstream of the common splice junction in exon II was first identified in adipose tissue and cells. All of these untranslated exons are spliced into the identical site upstream of the translation initiation site in exon II (Fig. 1). Consequently, the sequence encoding the open reading frame is identical in each case. Thus, the expressed protein is the same regardless of the splicing pattern.

In summary, it appears that three different 5′-termini are present at significant levels in human adipose. Of these, exon I.4 is present in breast adipose tissue and cultured adipose stromal cells in which aromatase is stimulated with dexamethasone in the presence of serum [34, 40]. Promoter II-specific sequences are present in cells stimulated with dibutyryl cAMP plus or minus phorbol ester in the absence of serum [35], but not in cells treated with dexamethasone. On the other hand, I.3 is present in adipose tissue and cells maintained under all conditions [40].

We also identified some *P*450arom mRNA species with other unique 5′-ends (*e.g.* exon I.2 in placenta) [40, 41]. However, these sequences are present in only a very small minority of *P*450arom transcripts in these tissues and will not be discussed further at this time.

BREAST CANCER AND ADIPOSE CYP19 GENE EXPRESSION

Quantification of breast adipose P450arom mRNA levels using competitive RT-PCR: an association between breast cancer localization and estrogen biosynthesis in breast adipose tissue surrounding a tumor

Levels of *P*450arom transcripts in adipose tissue are too low to be detected by northern analysis. We devised a specific competitive PCR amplification procedure coupled with reverse transcription (RT-PCR) to determine *P*450arom mRNA levels in breast adipose tissue [17, 24]. Although with PCR technology, specific low copy number mRNA species are readily amplified leading to excellent sensitivity, the exponential nature of amplification necessitates using an internal standard to reliably compare measured amounts of final products from one sample to another. For this purpose, a known amount of a homologous transcript may be coamplified as an internal control [42, 43]. We utilized reverse transcription and coamplification of a rat *P*450arom cRNA sequence (Fig. 2) in order to control and correct for the differences in amplification efficiency between samples [24, 30, 44].

In 10 out of 15 patients (67%), highest adipose *P*450arom transcript levels colocalized to the quadrants bearing tumors (Fig. 3). This correlation was statistically significant ($P < 0.001$). The regional distribution of *P*450arom transcripts in breast adipose tissue of disease-free individuals, obtained during reduction mammoplasty (control group, $n = 9$), did not favor any particular region of the breast. We also quantified by morphometry the histologic components of the adipose tissue samples from each quadrant in mastectomy specimens. The distribution of stromal cells significantly correlated with the distribution of *P*450arom transcript levels, in that quadrants containing highest proportions of stromal cells matched to highest transcript levels ($P < 0.01$). Although the quadrants bearing tumors contained highest numbers of stromal cells by parametric ANOVA, this correlation was at the level of statistical significance.

CLINICAL CONSIDERATIONS

Estrogen produced by adipose tissue within the breast may act locally to promote growth of breast tumors [45]. Estrogens can act both directly or in-

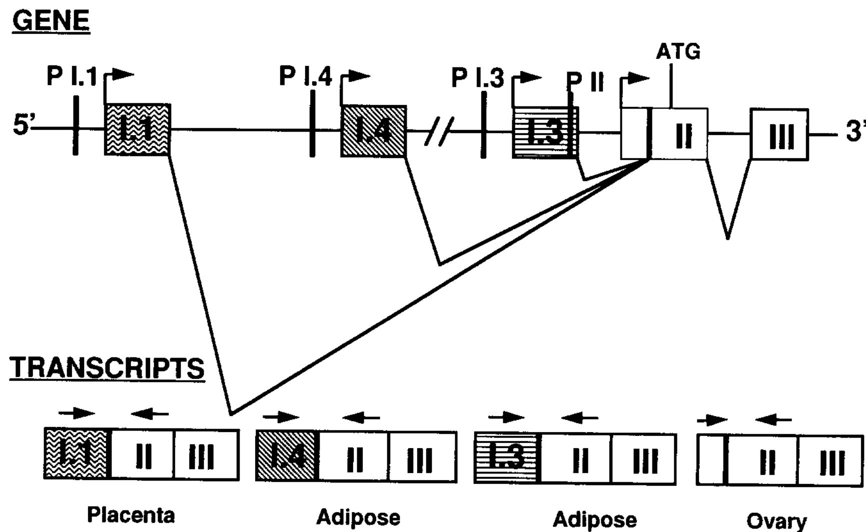

Fig. 1. Structure of the human CYP19 (*P*450arom) gene upstream of the translational start site, and of the transcripts present in placenta, adipose and ovary. The region encoding the *P*450arom protein contains 9 exons (II–X), of which only exons II and III are shown. Exon II contains the translation start site (ATG). A number of untranslated first exons are expressed in a tissue-specific fashion and encode the 5′ untranslated termini of *P*450arom mRNA in placenta (I.1) and adipose tissue (I.3 and I.4). These are spliced into the identical site upstream of the translation initiation site. In the ovary, transcription is started 120 bp upstream of the translation initiation site in exon II. The arrows above the transcript diagrams show the annealing sites of specific oligonucleotides employed for PCR amplification.

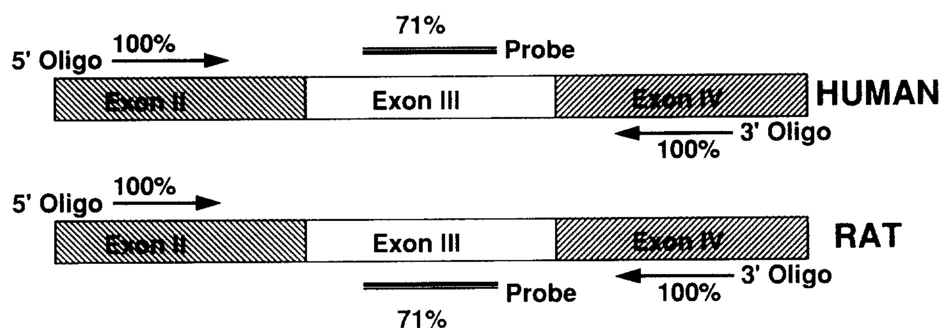

Fig. 2. Scheme for amplification of human and rat *P*450arom cDNAs. The human and rat aromatase cytochrome *P*450 cDNA sequences demonstrate an overall homology of 86%. The top sequence belongs to human whereas the bottom is rat. In the same sample, a 3′oligonucleotide is utilized for primer extension and PCR amplification of both human and rat cDNAs which recognizes a region showing complete identity in both species. The second primer in the PCR step is the 5′oligonucleotide which recognizes a region localized 272 bp upstream of the 3′oligonucleotide and these sequences are also identical between the two species. However, the two probes used for hybridization are designed to recognize heterologous portions (71% homology) of the cDNAs, and therefore are species-specific. And, by amplifying a coding region flanking 3 exons, amplification of any possibly contaminating genomic DNA was obviated.

directly on human breast cancer cells to promote proliferation. It is well established that breast cancer cells in culture elaborate a number of growth factors in response to estrogen, which can act in an autocrine and paracrine fashion to promote their proliferation and growth [46]. However, there is also evidence that estrogen can directly stimulate cellular proliferation [47]. Our recent findings suggest that locally produced estrogens may significantly influence neoplastic growth and development. Although several investigators have considered that aromatase activity of adipose tissue is too low to sustain meaningful levels of estrogen production, [48] it should be born in mind that aromatase is localized to specific cell types such as stromal cells [17]. Thus, determination of aromatase activity per unit weight of adipose would result in underestimation of the levels of aromatase activity present in specific tissue components, and hence of the levels of estrogen which might accumulate in local sites.

There are two possible non-exclusive mechanisms to account for increased *P*450arom transcripts in quadrants involved with tumor: (a) inherently higher production of estrogen positively influences tumor growth at that site; or (b) secretory products from tumors stimulate *P*450arom gene expression in the surrounding adipose tissue. Our data provides evidence that there is indeed an inherently determined distribution of *P*450arom gene expression in breast adipose tissue, as a consequence of the distribution of stromal cells versus adipocytes. This, in turn, may influence tumor growth. It should be pointed out that in these studies adipose tissue samples were consistently obtained at a fixed distance from the tumor, and the presence of micrometastases in the adipose tissue was excluded. Furthermore, there was no desmoplastic reaction in sampled fat. The range of differences between quadrants containing the lowest and the highest *P*450arom transcript levels within the same mastectomy specimen is 2- to 8-fold; whereas this range in tumor-free reduction mammoplasty specimens is 1.6- to 6-fold. The similarity in the variation in these two groups of patients supports the hypothesis that there is an inherently determined distribution of *P*450arom transcripts in breast regions independent of presence of a tumor. In addition, it appears that *P*450arom gene expression in neoplastic tissue samples varies extensively since *P*450arom transcript levels in tumors range from very high to very low [49].

Interestingly, in control mammoplasty specimens, highest *P*450arom transcript levels did not favor any particular quadrant unless a tumor was present. In mastectomy specimens, distribution of *P*450arom transcripts and the histologic components did not favor any particular quadrant, when tumor position was disregarded. By the same token, location of the tumor was also random. These findings do not support or contradict either hypothesis, namely that inherently higher production of estrogen in breast fat is the primary event, versus stimulation of aromatase activity in the surrounding fat by growth factors secreted by the tumor. Age range of our control group (reduction mammoplasty) was 20 to 40, whereas most breast cancer patients were postmenopausal. There was also a degree of variation in the fat content of the breast samples examined. Therefore, we were able to compare these two groups only with a certain level of caution.

Our results underscore the importance of aromatase inhibitors as efficacious agents in the endocrine treatment of hormone-responsive breast cancer [50]. Aromatase inhibitors effectively reduce local aromatase activity as well as blood estradiol levels [51]. Therefore, these agents will block estrogen delivery to breast cancer cells. Indirect support for the importance of local aromatase activity comes from studies correlating tumor aromatase activity with clinical responses to aromatase inhibition [52]. These considerations lend credence to the belief that a need exists for highly

specific and efficacious aromatase inhibitors for use in adjuvant therapy in breast cancer management.

Based on these considerations, we have proposed the following unified mechanism of oncogenesis in reference to the present and several previous studies. Regional differences in relative proportions of histologic components of the breast tissue (*e.g.* adipocytes, stromal cells) are the primary cause of estrogenic concentration gradients, since regions containing higher numbers of stromal cells are the sites of elevated *P*450arom transcript levels. Although the initiating events are unknown, once neoplastic cells start to replicate, tumor growth will be promoted by locally increased estrogen levels. Growth factors produced by

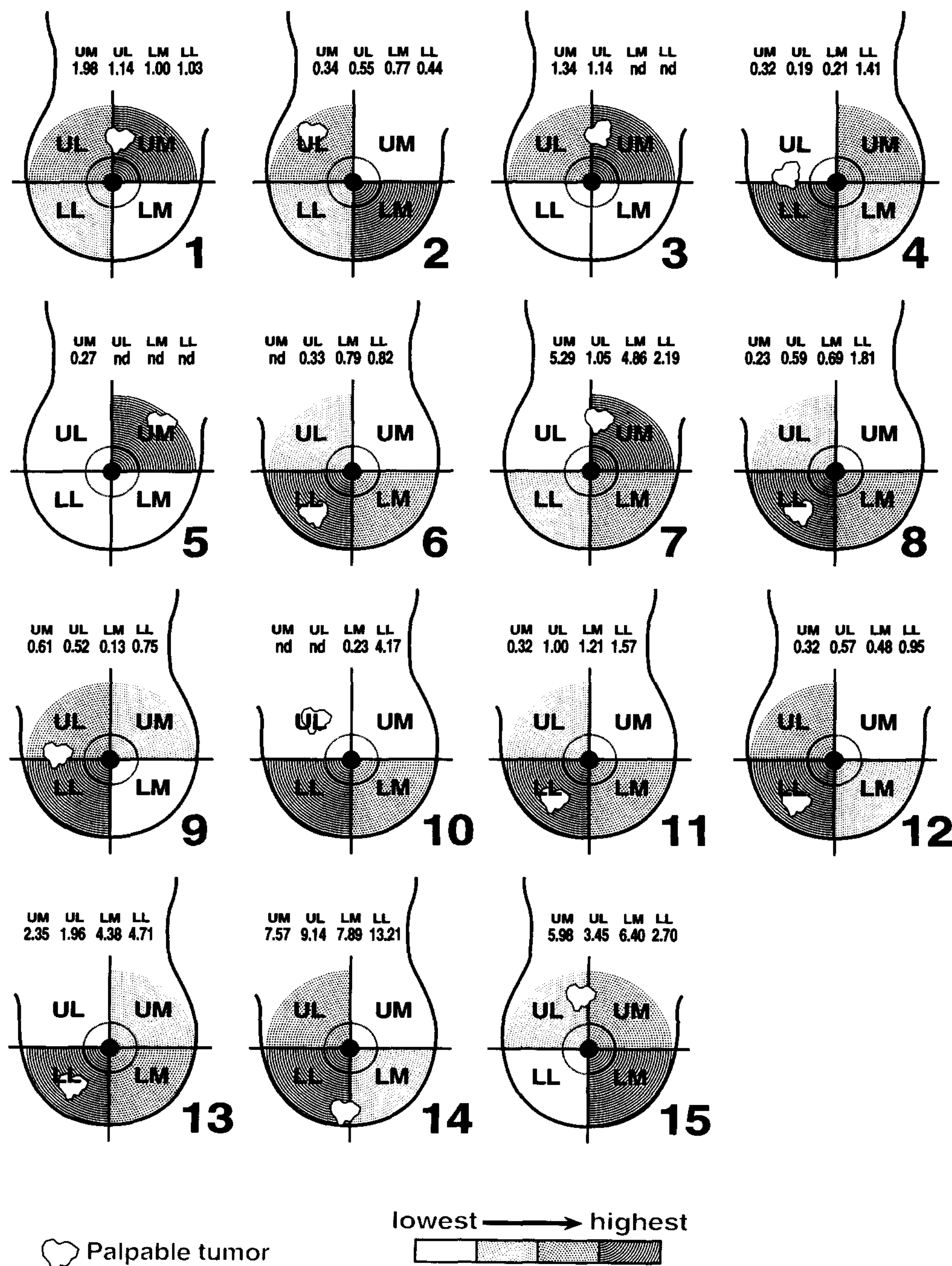

Fig. 3. In 10 out of 15 cases, highest *P*450arom transcripts were found in breast quadrants bearing a tumor. In 5 cases where the tumor occupied 2 adjacent quadrants, the index quadrant was chosen to be the one bearing the larger bulk of the tumor. *P*450arom transcripts were detected in all samples by autoradiography and in 46 out of 52 samples by the AMBIS Radioanalytic Imaging System. The lower-medial (LM) quadrant was never involved with tumor, while there was an equal distribution of involvement between upper-medial (UM, 4 out of 15), upper-lateral (UL, 5 out of 15) and lower-lateral (LL, 6 out of 15) quadrants. The numbers above each diagram represent *P*450arom transcript levels in quadrants expressed as corrected AMBIS readings as described in Fig. 3. (nd: not detected by AMBIS.)

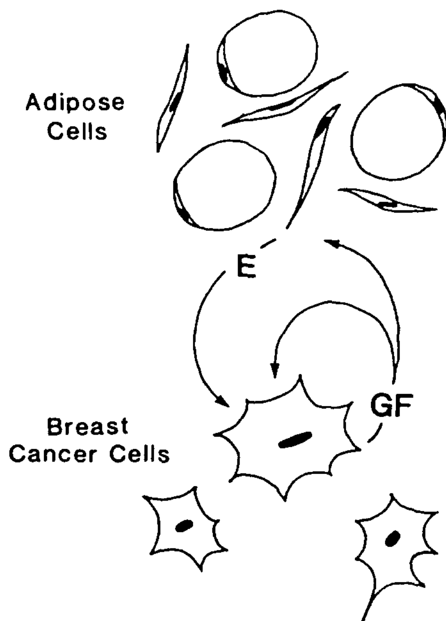

Fig. 4. Schematic representation of a proposed positive feed-back loop whereby estrogen produced locally by stromal elements of the breast adipose act to stimulate developing tumor cells to produce growth factors. These act in an autocrine fashion to stimulate growth and development of the tumor, and in a paracrine fashion to stimulate *P*450arom expression and hence estrogen biosynthesis, by the adipose stromal cells. E: estrogen; GF: growth factors. Reproduced from Simpson *et al.* (1989) with permission.

the tumor in response to estrogens may in turn further increase CYP19 gene expression in the surrounding adipose tissues (Fig. 4). At the same time, desmoplastic reactions to the presence of a tumor will cause proliferation of stromal-type tissue immediately proximal to the tumor, which will in turn sustain higher local estrogen levels. Estrogens will continue to positively influence neoplastic growth by increasing the expression of secretory products and their receptors in the tumor tissue. Thus a positive feed-back loop is established in which locally-produced estrogens and tumor-derived factors (*e.g.* growth factors) act by paracrine and autocrine mechanisms to sustain the growth and development of the tumor (Fig. 4).

SIGNIFICANCE OF THE IDENTIFICATION OF SPECIFIC TRANSCRIPTS OF HUMAN *P*450arom IN ADIPOSE TISSUE

Important immediate considerations include defining the molecular mechanisms responsible for maintaining elevated levels of *P*450arom transcripts in breast adipose tissue proximal to a tumor site. Recently, we have provided evidence for the use of alternative promoters to regulate *P*450arom expression in adipose tissue [39], and the differential regulation of the use of these promoters by growth factors and hormones [40]. Thus, breast adipose *P*450arom transcripts contain the newly-characterized 5′-terminus which we have termed untranslated exon I.4. This sequence may not be present in transcripts from lower body fat which instead contain another untranslated exon, namely exon I.3. (Both exons are present in transcripts from breast fat at varying degrees.) In adipose stromal cells, exon I.4 is uniquely present when the cells are cultured in the presence of serum plus glucocorticoids [40]. Under these conditions, *P*450arom expression is stimulated 20-fold as compared to controls [34], suggesting that sequences upstream of untranslated exon I.4 are regulated in a positive fashion by glucocorticoids and growth factors present in serum. By contrast, transcripts containing exon I.4 are absent when cells are maintained in the presence of cyclic AMP derivatives. Under these conditions, expression is markedly inhibited by serum and a number of growth factors [35, 36]. The fact that, in breast adipose tissue, *P*450arom expression is regulated by genomic elements which respond in a positive fashion to growth factors could provide a mechanism whereby growth factors produced by a tumor act on the surrounding adipose tissue to sustain the elevated synthesis of estrogens, and thus perpetuate a feed-back loop which drives the continuous development of the tumor (Fig. 4).

Finally, our results imply that monitoring gene expression of selected key proteins, such as *P*450arom, in certain tissues may prove to be clinically useful; and for this purpose, competitive RT-PCR as an alternate method to northern analysis may have more clinical applications in the near future. Moreover, the fact that CYP19 gene expression in breast adipose tissue is likely driven by a unique promoter, I.4, allows for the first time the possibility of developing drugs which can selectively inhibit estrogen biosynthesis uniquely in this tissue while permitting it to continue in other tissue sites, by selectively inhibiting CYP19 gene expression from this promoter region. In this way, it may be possible to develop adjuvant therapies for managing breast cancer patients employing tissue-selective estrogen withdrawal without subjecting them to a whole-body chemical castration, which essentially is the only possibility at the present time.

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