

Breast Cancer and the Role of Cytokines in Regulating Estrogen Synthesis: An Emerging Hypothesis

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I. Introduction

ESTROGENS have a central role in the development and growth of hormone-dependent breast tumors (1, 2), although the highest incidence of breast cancer occurs in postmenopausal women at a time when ovarian production of estrogen has ceased. However, the enzymes required for the peripheral synthesis of estrogens are present in other body tissues, such as adipose (3, 4), but also in most samples of normal and malignant breast tissues (5). While there was originally some controversy (6) as to whether the activities of these enzymes within breast tumors could result in the production of sufficient estrogen to exert a biological effect, *i.e.*, stimulate tumor growth, a number of factors have now been identified that can markedly enhance estrogen synthesis in breast cancer cells and fibroblasts derived from normal or malignant breast tissues. Recent advances in understanding the regulation of estrogen synthesis in the subclass of breast tumors that possesses the enzymes necessary for estrogen synthesis are considered in this review. Cytokines have emerged as being important regulators of estrogen synthesis in breast tissues, and a model is proposed for the involvement of the immune system and cytokines in controlling the peripheral synthesis of estrogens. For some time it has been known that peripheral estrogen formation is enhanced in obese or elderly subjects and that stress or immunosuppression may alter the risk of developing breast cancer. From the proposed model for the regulation of estrogen synthesis by cytokines, it is possible to suggest mechanisms to account for the effects of obesity and aging on estrogen synthesis and for the altered risks of breast cancer associated with stress or immunosuppression. A major objective of this review is to stimulate further research to support such a role for cytokines in the control of estrogen synthesis in breast cancer.

II. Peripheral Estrogen Synthesis and Blood and Tissue Estrogens in Breast Cancer

A. Peripheral estrogen synthesis

Peripheral estrogen synthesis results from the activities of three main enzymes. The aromatase enzyme complex, which converts adrenal androstenedione to estrone, is widely distributed throughout the body in adipose and muscle tissues (3, 7). Aromatase activity is also detectable in normal breast

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tissue and 40–50% of breast tumors (8, 9). Much of the estrone formed from androstenedione is converted to estrone sulfate by estrone sulfotransferase (10), and this estrogen conjugate can act as a reservoir for the formation of estrone via the action of estrone sulfatase (11, 12). Estrone is reduced to estradiol, the biologically active estrogen, by estradiol-17 β -hydroxysteroid dehydrogenase (Type I) (E2DH) (13, 14). The aromatase, estrone sulfatase, and E2DH Type I enzymes have all been isolated and their genes cloned (13–19). This has produced valuable information about their regulation at the molecular level (20, 21).

B. Aromatase

Soon after the discovery of the extraglandular route of estrone formation from androstenedione, it became apparent that the extent of this conversion was related to body weight (3, 22). In normal weight subjects about 1% of androstenedione is converted to estrone whereas in obese subjects this can increase up to 10%. The increase in the peripheral conversion of androstenedione to estrone associated with obesity most likely accounts for the increased risk that such subjects have for the development of endocrine-dependent cancers (23, 24).

In weight-matched subjects an increase in peripheral estrogen synthesis also occurs with aging (25). The increase in aromatase activity that occurs with aging, as detected from *in vivo* studies, was confirmed in one investigation in which adipose tissue aromatase activity was measured *in vitro* (26). In contrast, in another study an increase in *in vitro* aromatase activity was detected in adipose tissue taken from perimenopausal women (19.5 pg/mg/3 h) as compared with tissues from younger women (3.2 pg/mg/3 h) while that from menopausal women had conversion rates below 11 pg/mg/3 h (27). In a recent study, however, in which competitive RT-PCR analysis was used, levels of P450 aromatase transcripts in adipose tissue from buttocks, thighs, and abdomen of women were found to increase with advancing age (28).

An important finding to emerge from the first studies into the regulation of aromatase activity *in vitro* was the observation by Simpson and colleagues (29–31) that the synthetic glucocorticoid, dexamethasone, in the presence of FCS, could markedly stimulate aromatase activity. It was subsequently shown that the endogenous glucocorticoid, cortisol, could also stimulate *in vitro* aromatase activity (32). While glucocorticoids can stimulate aromatase activity *in vitro*, attempts to obtain evidence for such a role for glucocorticoids *in vivo* were not successful. The peripheral conversion of androstenedione to estrone in women given dexamethasone or Synacthen injections did not increase during therapy (33), with similar results being obtained in investigations carried out in monkeys (34). The reason for the failure of glucocorticoids to enhance *in vivo* aromatase activity remains puzzling but will be discussed later in the review in the light of current knowledge of the control of aromatase activity.

C. Estrone sulfatase

Blood and breast tissue concentrations of estrone sulfate are much higher than for the unconjugated estrogens (35–37)

and, furthermore, the half-life of estrone sulfate (10–12 h) in blood is much longer than for unconjugated steroids (20–30 min) (38). Estrone sulfate may therefore act as a reservoir for the formation of estrone after hydrolysis by estrone sulfatase (11, 12). The activity of estrone sulfatase is much higher than the aromatase in normal and malignant breast tissues and, in contrast with the aromatase, is present in most breast tumors (5). Using physiological substrate concentrations, formation of estrone via the sulfatase pathway was found to account for a 10-fold greater amount of breast tumor estrone than that formed via the aromatase route (39). In rat nitrosomethylurea-induced mammary tumors, a model used to investigate hormone-dependent tumors, up to 50% of the estrone was found to originate *in situ* from the hydrolysis of estrone sulfate (40).

D. E2DH

Estrone, formed from either androstenedione or estrone sulfate, is converted to estradiol by E2DH. It was originally thought that E2DH, as such, was responsible for the interconversion of estrone and estradiol and that it could act in either an oxidative or reductive direction dependent upon cofactor availability. However, infusions of either [³H]estrone or [³H]estradiol in women with breast tumors revealed that within tumors little metabolism of [³H]estradiol occurred whereas [³H]estrone was readily converted to [³H]estradiol (41). For benign and malignant breast lesions a positive correlation was found between E2DH activity in adipose tissue surrounding the breast lesion and the degree of obesity of the individual (42). It is now apparent that E2DH (Type I) is present in breast tumors and that this dehydrogenase is responsible for the reduction of estrone to estradiol (13, 14).

E. Origin of breast tumor estrogens

In contrast to the low levels of estrogens found in the plasma of postmenopausal women, preliminary measurements of breast tumor estrogen concentrations, using a mass fragmentography technique, initially indicated that breast tumors may contain high estradiol concentrations (43). Several investigations, using fully validated RIA techniques, have now compared plasma estrogen concentrations with those in normal and malignant breast tissues. A consensus has emerged from these investigations indicating that the concentrations of estrone and estradiol in both normal and malignant breast tissues are significantly higher than the levels in plasma. Furthermore, estrogen concentrations, and in particular estradiol, are higher in malignant than in normal breast tissue (43–47). A consistent finding from these investigations was that the tumor-plasma estradiol ratio (up to 20-fold) was much higher than that for estrone (44, 45). One intriguing finding to emerge from measurements of breast tissue estrogens was the observation that while tissue estrone concentrations in postmenopausal women reflected the decrease in estrogen production that occurs at menopause, tumor estradiol concentrations were independent of menopausal status with similar levels being detected in tumors from pre- and postmenopausal women (48–50).

As estrogens in breast tumors could originate via uptake from the circulation, the estradiol content of estrogen receptor positive (ER+) or ER negative (ER-) tumors were compared. While some evidence was obtained for higher estradiol concentrations in ER+ than in ER- tumors (51–53), no consistent correlation was detected between ER and estradiol content as might be expected if uptake and retention was the major source of tumor estradiol (45, 47, 52).

An alternative and more likely explanation is that since the enzymes necessary for estradiol synthesis are present in most breast tumors, *in situ* formation makes a major contribution to the high estradiol content of these tumors. In an attempt to obtain information on the origin of estrogen found in breast tumors, a double isotopic infusion technique was developed to allow uptake of estrogen from the circulation to be differentiated from *in situ* estrone synthesis in normal and malignant breast tissues (54). In some breast tumors there was little evidence of *in situ* estrone synthesis, but in others up to 90% of the estrone content of tumors was identified as resulting from *in situ* synthesis. Furthermore, significant *in situ* estrone formation in normal breast tissue was detected only in tissue adjacent to tumors actively synthesizing estrogen (54).

III. Influence of Breast Tumor Location on Enzyme Activities in Adjacent Tissues

Results obtained from *in vivo* and *in vitro* studies had indicated that in breast tumors, estrone was preferentially converted to estradiol and that the activities of the aromatase, E2DH, and estrone sulfatase enzymes were higher in malignant than normal breast tissues (5, 54). These findings, together with the evidence of increased breast tumor estradiol concentrations, led to the suggestion that breast tumors may contain or produce factors that could influence not only the direction of estrogen metabolism within tumors but also stimulate the activities of the enzymes involved in estrogen synthesis (55).

Support for this concept was provided by results from a study that found a highly significant correlation between the size of malignant, but not benign, breast tumors and E2DH activity in adipose tissue adjacent to the tumor (56). E2DH activity within breast tumors was also found to correlate with activity in normal breast tissue taken some 2–5 cm from the tumor (57).

Similar investigations revealed that the location of a tumor within the breast could also influence aromatase activity in the breast quadrant in which the tumor was located. Miller and his colleagues (58, 59) made the important observation that aromatase activity in the tumor-bearing breast quadrant was always higher than in non-tumor-bearing quadrants. This finding has now been confirmed in two further studies of mRNA expression (60) and tissue activity level (61), and only one other study produced conflicting results (62). Therefore, results from three independent investigations have confirmed that the location of a tumor within the breast is able to influence aromatase expression and activity in adjacent tissues.

Some evidence that estrone sulfatase activity may also be influenced by tumor location has also been obtained, but further investigations are required to confirm the significance of this observation (63).

IV. Proposed Model for the Regulation of Estrogen Synthesis in Breast Tumors

The observations that the location of a tumor within the breast could influence aromatase, E2DH, and estrone sulfatase activities in adjacent tissues stimulated an intensive, on-going search to identify the nature and cellular origin of the stimulatory factors. From this research it has been possible to develop a model to account for the regulation of estrogen synthesis in breast tumors in which cells of the immune system have an important role as a major source of the factors that stimulate tumor estrogen synthesis. The proposed model, which is shown in Fig. 1, will first be described with the supporting evidence discussed in detail later in the review.

Breast tumor cells exist in a complex matrix of other cells that includes stromal cells and adipocytes. However, it is now evident that a substantial proportion (up to 50%) of breast tumors can be comprised of cells of the immune system that infiltrate tumors. These include the tumor-associated macrophages (TAMs) and tumor-infiltrating lymphocytes (TILs) that are attracted by chemokines, such as interleukin (IL)-8 and macrophage chemoattractant protein-1 (MCP-1) secreted by tumor cells. A plentiful blood supply is an essential requisite for tumor growth, and there is now good evidence that TAMs may be an important source of angiogenic factors that stimulate blood vessel development in breast tumors (64).

As previously noted, while only 40–50% of breast tumors possess aromatase activity (9), most tumors have highly active estrone sulfatase and E2DH (Type I) enzyme systems (5). Immunocytochemical studies to examine the location of aromatase in breast tumors have provided evidence for a stromal (65, 66) and epithelial (67, 68) location for this enzyme complex. Measurements of aromatase activity in stromal tumor-derived fibroblasts or epithelial MCF-7 breast cancer cells have revealed that a much higher level of aromatase activity is present in stromal cells (69). Immunocytochemical analysis of the location of E2DH has revealed that the enzyme is located in the cytoplasm of malignant epithelial cells (70).

Cytokines, and in particular IL-6 and tumor necrosis factor- α (TNF α), have emerged as having crucial roles in regulating estrogen synthesis in breast cancer cells. IL-6 and TNF α both stimulate aromatase, E2DH, and estrone sulfatase activities and furthermore can also act synergistically to enhance the activities of these enzymes. While IL-6 can be secreted by breast tumor-derived fibroblasts and macrophages, a major source of IL-6 produced within breast tumors is thought to be the infiltrating lymphocytes.

The ability of IL-6 to stimulate aromatase activity can be markedly potentiated by the IL-6 soluble receptor (IL-6 sR). IL-6 sR is produced by malignant epithelial cells and fibroblasts derived from malignant, but not normal, stro-

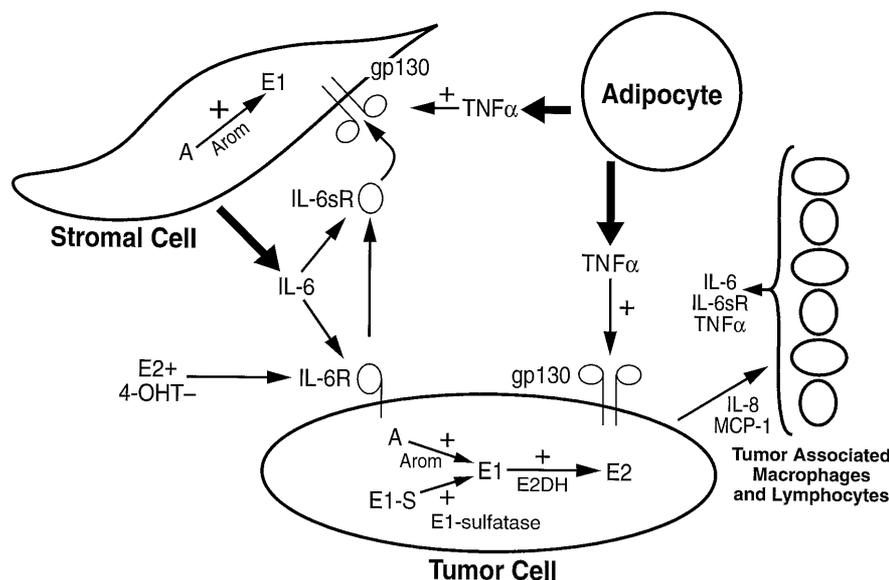


FIG. 1. Proposed model for the regulation of estrogen synthesis in breast tumors. Breast cancer cells are embedded in a matrix of stromal cells and adipocytes with up to 50% of the volume of a tumor being comprised of TAMs and TILs. In epithelial and stromal cells androstenedione (A) is converted to estrone (E1) by the aromatase (arom) enzyme complex. Formation of E1 from estrone sulfate (E1S) by estrone sulfatase (E1-sulfatase) is also an important source of tumor estrogen, with estrone being converted to estradiol by E2DH Type I. TAMs and TILs invade tumors in response to chemokines, such as IL-8 and MCP-1, secreted by tumor cells. The cytokines IL-6 and TNF α , which are secreted by cells of the immune system and also by stromal cells (IL-6) and adipocytes (TNF α), have a central role in regulating tumor estrogen synthesis. The ability of IL-6 to stimulate estrogen synthesis can be potentiated by TNF α , probably as a result of TNF α increasing the expression of the gp130 protein that is involved in the IL-6-signaling pathway. The IL-6R can also exist in a soluble form (IL-6 sR) and can potentiate the ability of IL-6 to stimulate aromatase activity. Shedding of IL-6 sR is increased by estradiol (E₂) with the E₂-stimulated increase being inhibited by the antiestrogen, 4-hydroxytamoxifen (4-OHT). The gp130 protein is ubiquitously expressed, and it is possible that IL-6 in association with the IL-6 sR can induce an IL-6 response in tumor-adjacent cells lacking the IL-6R. Overall cytokines coordinate an increase in estrogen synthesis which may account for the high E₂ content of most breast tumors.

mal cells, but again, a major source of IL-6 sR within breast tumors is from the TAMs and TILs. Shedding of IL-6 sR from malignant epithelial cells can be increased by estradiol with the antiestrogen, tamoxifen, being able to block the estradiol-stimulated increase in shedding. The IL-6 receptor (IL-6R) and/or IL-6 sR can interact with the gp130 component of the IL-6R system, and this is required for the induction of signal transduction that results from IL-6 binding to its receptor. As the gp130 protein appears to be expressed ubiquitously, it is possible that IL-6, in association with its soluble receptor, could stimulate aromatase activity in other cells within the body not expressing the IL-6R. It is also likely, but remains to be shown, that IL-6 in combination with the IL-6 sR, may also enhance E2DH activity in breast cancer cells, although there is indirect evidence for such an interaction. TNF α , which is secreted by adipocytes and cells of the immune system, can increase expression of the gp130 protein component of the IL-6R-signaling system, and this probably accounts for the synergy that occurs between IL-6 and TNF α in their ability to stimulate estrogen synthesis. Overall, it has emerged that there is a complex system of regulating estrogen synthesis within breast tumors that appears to be mainly coordinated by cytokines. The result of the coordinated stimulation of estrogen synthesis may account for the high concentrations of estradiol found in breast tumors.

V. Evidence in Support of Proposed Model of Cytokine Regulation of Estrogen Synthesis

A. Identification of factors that stimulate estrogen synthesis

To obtain evidence to support the concept that breast tumors produced factors that stimulated estrogen synthesis, initial studies investigated the interaction between breast tumor homogenates and E2DH activity in adipose tissue explants (71). Using this system, conversion of estrone to estradiol was stimulated by breast tumor homogenates while no effect on the oxidative metabolism of estradiol was detected. It was subsequently shown that cytosol prepared from breast tumors, but not normal breast tissue, could also preferentially stimulate E2DH reductive (*i.e.*, formation of estradiol from estrone) activity in MCF-7 breast cancer cells (72) and aromatase activity in cultured breast tumor-derived fibroblasts in the presence of dexamethasone (73). Breast cyst fluid (BCF), which is obtained from breast cysts that are common in premenopausal women (74, 75), and which may be associated with an increased risk of breast cancer (75, 76), was also found to be able to stimulate aromatase and E2DH activities in cultured breast cells (73, 77–80).

Analysis of breast tumor cytosol and BCF led to the identification of several factors that could stimulate estrogen synthesis, including the insulin-like growth factors, Types I and II (81). An albumin-like factor was also identified in

breast tumor cytosol (82). Some types of human serum albumin were subsequently shown not only to stimulate E2DH and aromatase activities but also to potentiate the ability of growth factors, such as IGF-I, or cytokines, such as IL-1, to stimulate the activities of these enzymes (73, 83) (Fig. 2). The ability of IL-1 to interact with human serum albumin was examined as it had previously been reported that IL-1-like activity was associated with BSA (84). In view of the proposed model of the regulation of estrogen synthesis by cytokines, it is of interest that some types of albumin have recently been reported to increase production of IL-6 and PGE₂ by macrophages (85). It is possible that a modified form of albumin in breast tumors may have a similar immunostimulatory role.

The cytokines IL-1 and IL-6 were detected in BCF by RIA analysis, and both cytokines were found to stimulate aromatase activity in breast tumor-derived fibroblasts in the presence of dexamethasone (79, 86). However, the concentration of IL-6 in BCF was 1000-fold higher than that of IL-1, suggesting that IL-6 was the major aromatase stimulatory factor present in BCF.

IL-6 was also identified in conditioned medium (CM) from cultured breast tumor-derived fibroblasts as one of the factors responsible for the ability of this medium to stimulate E2DH reductive activity in MCF-7 breast cancer cells (87–89). Recombinant IL-6 (rIL-6) also stimulated E2DH activity in these cells although its ability to increase enzyme activity was not confirmed in another investigation (90). The degree to which rIL-6-stimulated E2DH activity was lower than that achieved by CM containing a comparable concentration of IL-6, suggesting that other factors may also be present in the CM and able to potentiate the stimulatory effect of IL-6 on E2DH activity. Since

TNF α is produced by adipocytes (91, 92), and with adipose tissue forming a substantial proportion of tissue in the breasts of postmenopausal women, a combination of TNF α and IL-6 was tested for its ability to stimulate E2DH activity. TNF α markedly potentiated the ability of IL-6 to stimulate E2DH activity although *in vitro* this combination of cytokines inhibited cell proliferation (90). TNF α also stimulates estrone sulfatase activity in MCF-7 cells and aromatase activity in fibroblasts derived from normal and malignant breast tissues, with TNF α and IL-6 also acting synergistically to enhance the activities of these enzymes (93, 94) (Fig. 3). Other cytokines that are members of the IL-6 superfamily, including IL-11, oncostatin M, and leukemia inhibitory factor, can also increase aromatase activity in cultured adipose tissue stromal cells (95).

CM collected from MDA-MB-231 breast cancer cells is also capable of stimulating aromatase activity in cultured adipose tissue-derived stromal cells (96). The stimulatory factor in CM collected from MDA-MB-231 cells eluted from an ion-exchange column at a similar concentration of sodium chloride to that used to elute IL-6 in CM from tumor-derived fibroblasts. Therefore, it is likely that this stimulatory factor will prove to be IL-6 (73). The ability of MDA-MB-231 cells to produce IL-6 was recently reported (97).

Several different factors that can stimulate estrogen synthesis in cultured breast cancer cells or adipose tissue stromal cells have now been identified in breast tumor cytosol, BCF, and CM from breast tumor-derived fibroblasts. However, it is apparent that cytokines, and in particular IL-6 and TNF α , are emerging as the primary factors regulating aromatase (93), E2DH (89, 90), and estrone sulfatase (94) activities.

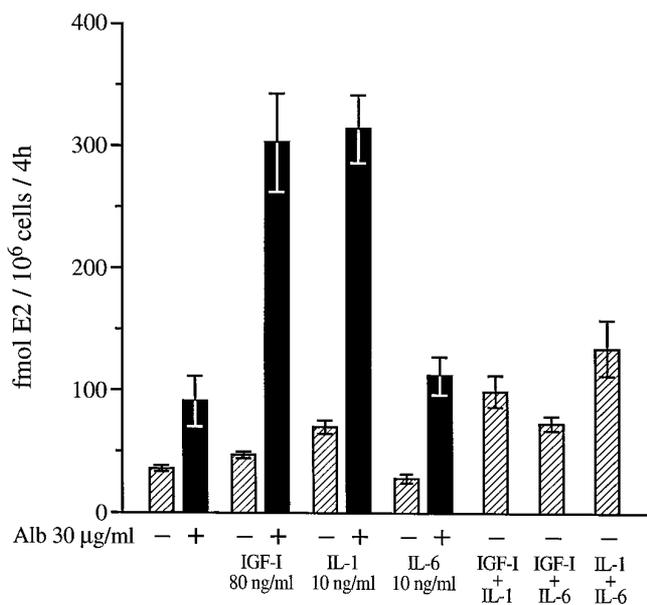


FIG. 2. Potentiation of growth factor and cytokine stimulation of estradiol 17 β -hydroxysteroid dehydrogenase (reductive) activity by human serum albumin in MCF-7 breast cancer cells. [Reproduced from A. Singh *et al.*: *Mol Cell Endocrinol* 85:165–173, 1992 (83) with kind permission from Elsevier Science Ireland Ltd., Bay 15K, Shannon Industrial Estate, Co. Clare, Ireland.]

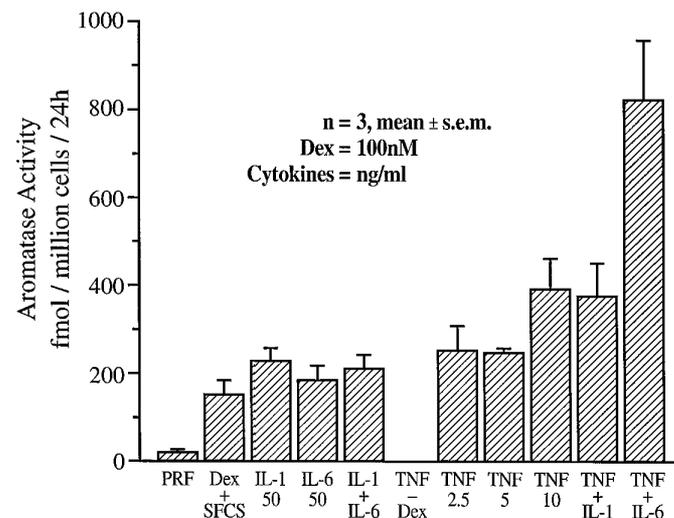


FIG. 3. Stimulation of aromatase activity by cytokines in fibroblasts derived from normal breast tissue from a premenopausal woman. Phenol red-free (PRF) medium had little effect on aromatase activity whereas dexamethasone plus stripped FCS (SFCS), used as a positive control, increased aromatase activity. Cytokines, when tested in the presence of dexamethasone, but absence of SFCS, markedly increased aromatase activity with IL-6 and TNF α acting synergistically. [Reproduced with permission from A. Purohit *et al.*: *Endocr Rel Cancer* 4:323–330, 1997 (94).]

B. Potentiation of cytokine stimulation of estrogen synthesis

While cytokines can stimulate aromatase, E2DH, and estrone sulfatase activities in breast cancer cells and fibroblasts, the extent of stimulation by a single cytokine is usually relatively modest. The finding that some human serum albumin preparations markedly potentiated the ability of cytokines and growth factors to stimulate *in vitro* aromatase activity (73) suggested that such a mechanism may also be effective in breast tumors.

Further evidence in support of the potentiation of cytokine stimulation of enzyme activity was provided by a comparison of the effects of recombinant cytokines, such as IL-6, and CM from tumor-derived fibroblasts in which the concentration of IL-6 was measured. While there is no doubt that such CM does contain IL-6, the initial report that rIL-6 stimulates E2DH activity was difficult to confirm (90). In the initial study it was apparent that whereas CM containing IL-6 at a concentration of 2 ng/ml resulted in a 250% stimulation of E2DH reductive activity, rIL-6 at the much higher concentration of 80 ng/ml stimulated activity by only 150% (89). In another study CM containing IL-6 at a concentration of 5 ng/ml stimulated E2DH reductive activity almost 1000-fold, whereas rIL-6 from several different sources had only a modest effect on the activity of this enzyme (90). It was concluded from this investigation that in addition to IL-6, other factors, such as other cytokines and/or proteins, must also be present in CM from tumor-derived fibroblasts. Such factors must be able to greatly potentiate the ability of cytokines, such as IL-6, to stimulate enzyme activity.

Similar differences in the ability of cytokines to stimulate aromatase activity have also been noted. The initial finding that IL-6 could stimulate aromatase activity was detected using tumor-derived fibroblasts (79). In contrast, using stromal cells derived from subcutaneous adipose tissue, Simpson and his colleagues were initially unable to confirm an effect of IL-6 on aromatase activity (personal communication). It seemed possible, therefore, that the difference in the ability of fibroblasts derived from breast tumors and stromal cells derived from adipose tissue may depend upon the secretion of a coregulatory protein by the former cells.

Simpson and his colleagues (95) recently showed that the combination of IL-6 with its soluble receptor (IL-6 sR) resulted in a marked stimulation of aromatase activity in stromal cells derived from subcutaneous adipose tissue. The ability of IL-6 sR to potentiate the stimulatory effect of IL-6 on aromatase activity in fibroblasts derived from subcutaneous adipose tissue was recently confirmed (98). Significant aromatase activity was detectable in IL-6-stimulated fibroblasts, but the combination of IL-6 sR plus IL-6 resulted in a 21-fold greater stimulation of activity than with IL-6 alone (Fig. 4).

Thus it appears likely that differences in the ability of rIL-6 and CM from tumor-derived fibroblasts containing IL-6 and the ability of different fibroblasts to respond to IL-6 depends upon the presence of IL-6 sR or the ability of cells to secrete IL-6 sR.

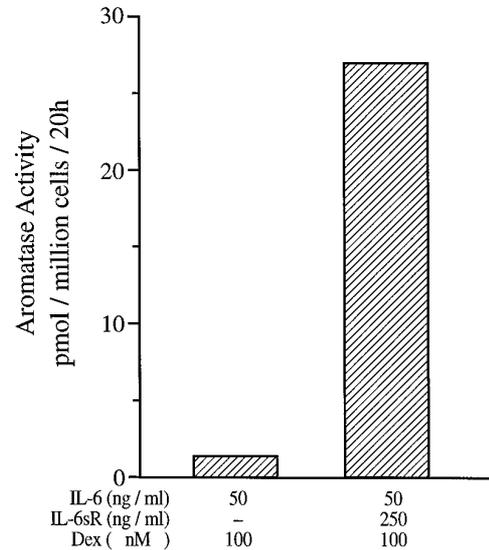


FIG. 4. Effect of IL-6 or IL-6 plus IL-6 sR on aromatase activity in primary cultures of dexamethasone-stimulated fibroblasts derived from subcutaneous adipose tissue. Results are the means of triplicate experiments for which the variation was <10%. [Reproduced from A. Singh *et al.*: *J Endocrinol* 147:R9–R12, 1995 (98) by permission of the Journal of Endocrinology Ltd.]

VI. The Role of Soluble Cytokine Receptors in Cytokine Action

A. Mechanism of cytokine action

Cytokines act by binding to membrane spanning receptors (99, 100). The IL-6R complex consists of an 80-kDa (gp80) ligand-binding subunit and a 130-kDa (gp130) signal-transducing protein (101). The small gp80 subunit binds IL-6 with low affinity and must associate with the larger gp130 in order for high-affinity binding and signal transduction to occur (102). A 55-kDa soluble form of gp80 (IL-6 sR) has also been found in high concentrations in urine and serum (103, 104). Unlike all other known soluble cytokine receptors, which antagonize the effects of their respective cytokines, IL-6 sR enhances the response to IL-6 in some biological systems. The IL-6 sR is formed by limited proteolysis (shedding), but the proteinase responsible for the formation of IL-6 sR has not yet been identified. It does not appear to belong to one of the classic groups of proteolytic enzymes (105). Phorbol esters that activate the protein kinase C signaling pathway are potent inducers of IL-6 sR shedding (106), although the physiological stimulus for protein kinase C activation that results in receptor shedding is not yet known. Recent evidence has indicated that the IL-6 sR may also be formed by an alternative splicing of mRNA, leading to loss of the transmembrane domain (107).

The combination of IL-6 plus IL-6 sR has been shown to enhance the secretion of acute phase proteins by Hep G2 cells (108) and the inhibition of growth of T47D breast cancer cells (109), compared with the effects of IL-6 alone. It is likely, therefore, that by the shedding of IL-6 sR by one cell type, and after ligand binding, the complex could act on cells that only express gp130 at their surface (99). Such cells would not normally react to IL-6, but it is known that gp130 is present

on all cell types whereas the IL-6R is not expressed ubiquitously. Using a cytokine gene transfer-based tumor rejection model, IL-6 sR was also shown to be active *in vivo* (110).

B. Regulation of gp80 and gp130

Recent studies have provided important insight into the regulation of gp80 and gp130. IL-6 at high concentrations down-regulates IL-6R (gp80), resulting in cells becoming desensitized (108). Glucocorticoids regulate IL-6R (gp80) expression, but their effects differ for different cell types. Treatment of monocytes with dexamethasone results in down-regulation of IL-6R (gp80) mRNA expression (111), whereas in hepatocytes and epithelial cells glucocorticoids have a positive effect on gp80 mRNA expression (112). IL-6 can increase the expression of gp130 mRNA to a small extent, whereas expression is markedly up-regulated by a combination of IL-6 and dexamethasone in Hep G2 cells (113). In human UAC (U Amnion Cell) and Hep 3B (hepatoma) epithelial cells, in addition to IL-6, IL-1 and TNF α also increase expression of gp130 mRNA (114). The increase in gp130 expression caused by TNF α is the most likely explanation for the marked synergy seen between IL-6 and TNF α in their ability to stimulate estrogen synthesis in breast cancer cells.

C. Regulation of IL-6 sR shedding

In a recent investigation, the effects of steroids, cytokines, or 12-*O*-tetradecanoyl phorbol-13-acetate on the release of IL-6 sR from MCF-7 breast cancer cells was examined (98). Treatment of cells with estradiol resulted in a marked increase in the release of IL-6 sR, and this estradiol-stimulated release was almost completely abolished by the antiestrogen, 4-hydroxytamoxifen. Both IL-6 and TNF α increased IL-6 sR release, as did dexamethasone and 12-*O*-tetradecanoyl phorbol-13-acetate. IL-6 sR was also detected in CM from another ER+ breast cancer cell line, T47D, but not in the ER- MDA-MB-231 cell line, a result that was recently confirmed (97). IL-6 sR was also detected in CM collected from malignant tumor-derived fibroblasts but not from benign or normal breast tissue-derived fibroblasts (98). CM from lipopolysaccharide-stimulated macrophages and lymphocytes also contained high concentrations of IL-6 sR. Concentrations of IL-6 sR were higher in cytosol prepared from malignant breast tissue than in cytosol from normal breast tissue. The finding of significant concentrations of IL-6 sR in fibroblasts derived from malignant but not normal (mainly adipose) breast tissue offers a ready explanation for the discrepancy in the ability of IL-6 alone to stimulate aromatase activity in cells derived from these different tissues (73, 95). The ability of estradiol to increase the release of IL-6 sR, and inhibition of this stimulation by 4-hydroxytamoxifen, could also account for the effects that these compounds have on estrogen synthesis in breast cancer cells. Estradiol markedly potentiates the ability of IL-6 to stimulate E2DH reductive activity in MCF-7 cells (115), while tamoxifen inhibits the ability of IL-6 to stimulate enzyme activity (116).

VII. Origin of Estrogen-Stimulating Factors in Breast Tumors

A. The role of cells of the immune system

While there is evidence that cytokines, such as IL-6, are produced by breast tumor-derived fibroblasts, it has recently become evident that macrophages and lymphocytes that invade tumors are also likely to be a major source of tumor cytokine production. Although the roles that macrophages and lymphocytes may have as sources of cytokines that stimulate estrogen synthesis are still being investigated, their potential role as stimulators of tumor growth has been studied for many years (117–120). Important clues to the fact that cells of the immune system might have a role *in vivo* in modulating breast tumor estrogen synthesis were provided as a result of two clinical investigations. The inhibitor, 4-hydroxyandrostenedione, was found to effectively abolish peripheral aromatase activity, as measured in the whole body using infusion of isotopically labeled steroids (121). In the same study, tumor biopsies were obtained before and after therapy with 4-hydroxyandrostenedione and aromatase activity was measured in these specimens *in vitro*. While the majority of samples examined after inhibitor therapy showed a significant decrease in aromatase activity, for two subjects a large increase in *in vitro* aromatase activity was detected. DNA polymerase α activity was also measured in these biopsy samples, as a marker of cell proliferation, and in the two specimens showing an increase in aromatase activity there was a corresponding increase in DNA polymerase activity. This was one of the first findings to suggest that breast tumor aromatase might be capable of producing a biological effect, *i.e.*, produce sufficient estrogen to induce cell proliferation. Further support for this concept was provided by other observations arising from this study (54). A marked decrease in *in situ* tumor estrone synthesis was associated with a decrease in tumor estrone concentrations and an increase in cell nuclear condensation, a marker of apoptosis (122, 123). A similar result showing an increase in breast tumor aromatase activity, when measured *in vitro* after 4-hydroxyandrostenedione therapy, was also obtained in another study (124).

At the time it was difficult to comprehend what factors might be capable of stimulating tumor aromatase activity in the presence of 4-hydroxyandrostenedione that effectively blocked peripheral aromatase activity. The wound healing that occurs after obtaining a biopsy sample would be associated with macrophage and lymphocyte invasion. It is possible that infiltration by these cells of the immune system may have provided sufficient cytokines to stimulate aromatase activity in these two samples, even in the presence of 4-hydroxyandrostenedione.

A further indication that macrophages and lymphocytes may have an important role in regulating tumor aromatase activity came from a study of aromatase activity in breast tissue obtained from a woman who had previously undergone breast augmentation by silicone injection, which was not contained within a capsule (61). In normal breast adipose tissue, aromatase activity is relatively low (~10 fmol/mg

protein/3 h) (5). For the subject who had had breast augmentation, aromatase activity of up to 400 fmol/mg protein/3 h was detected (61). Histological examination of this tissue revealed the presence of inflamed tissue with extensive macrophage and lymphocyte invasion. Furthermore, a significant correlation was found between aromatase activity in samples of this breast tissue and the ability of tissue explants to produce IL-6, although the cells in the tissue responsible for IL-6 production were not identified.

B. Macrophages and lymphocytes in breast tumors

There is evidence that as much as 50% of the volume of breast tumors is comprised of macrophages and lymphocytes (125), and it is therefore likely that such cells are the major source of cytokines that are present in tumors and available to stimulate estrogen synthesis. The finding of a strong association between oncogene amplification and dense lymphocytic infiltration of tumors lends additional support for an important role for these cells in regulating tumor growth (126). Evidence to support such a role for macrophages and lymphocytes was obtained by collecting CM from lipopolysaccharide-stimulated peripheral blood monocytes and lymphocytes. In the presence of dexamethasone, CM from macrophages and lymphocytes stimulate aromatase activity to a greater degree than ever previously detected (61). CM from these cells also stimulate E2DH reductive and estrone sulfatase activities in MCF-7 breast cancer cells (127).

Breast tumor cells are known to secrete a number of chemokines, such as IL-8 and MCP-1, which attract both macrophages and lymphocytes (128–131). Epithelial cells derived from breast tumors have recently been shown to secrete large amounts of the chemokine IL-8 (132).

Thus, in addition to the possibilities of breast tumors producing factors that might stimulate estrogen synthesis in adjacent tissues, or that enhanced estrogen synthesis in normal breast tissue may favor tumor development, it is also possible that increased estrogen synthesis in tumors and adjacent tissues results from macrophage and lymphocyte invasion. Further support for a three-component model (*i.e.*, epithelial, stromal, and immune cells) as being important in regulating intratumoral estrogen synthesis has recently been put forward (133).

The attraction of macrophages and lymphocytes to the margins of a tumor could account for the original observation of a significant correlation between tumor size and E2DH activity in tissue adjacent to the tumor. As shown in Fig. 5, the correlation between E2DH activity in tissue adjacent to the tumor and tumor size (56) (Fig. 5A) is very similar to the correlation relating CD4⁺ invasion and tumor size (134) (Fig. 5B). Similarly, the presence of a greater number of macrophages and lymphocytes in the breast quadrant bearing a tumor, with the subsequent release of aromatase-stimulatory cytokines, is the most likely explanation for the consistent finding of an association between tumor location and breast adipose tissue aromatase activity.

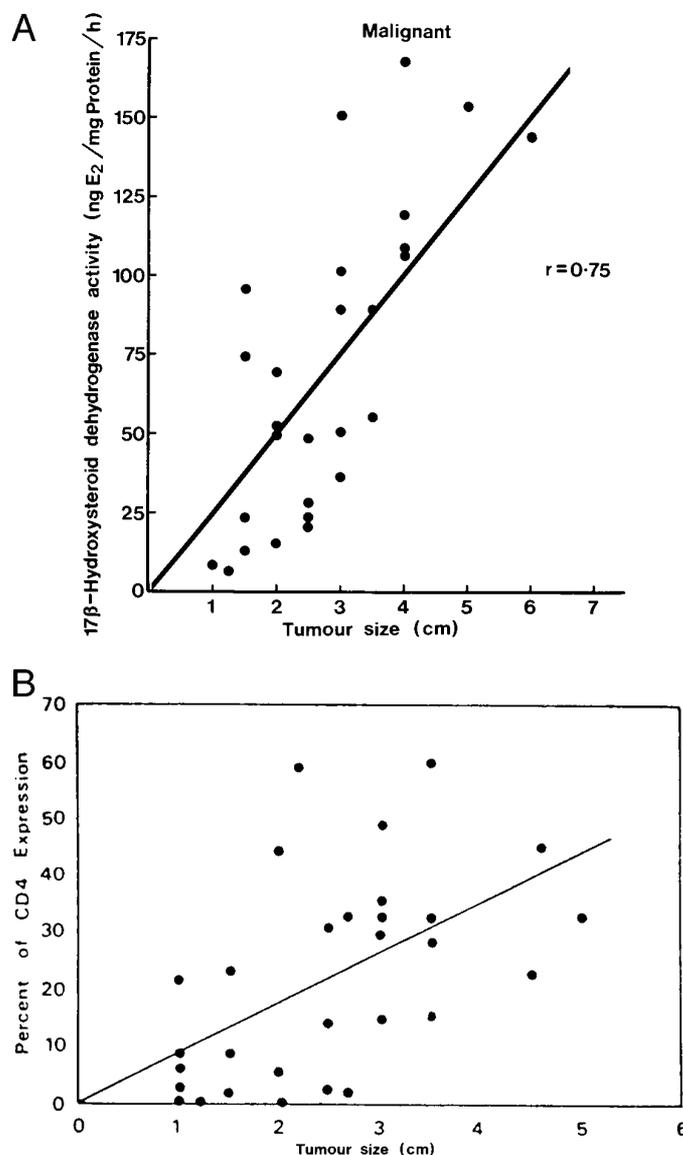


FIG. 5. Similar correlations found between: A, Estradiol 17 β -hydroxysteroid dehydrogenase activity in tissue adjacent to breast tumors and tumor size ($r = 0.75$, $P < 0.001$); and B, CD4⁺ (T lymphocyte) infiltration of tumors and tumor size ($r = 0.75$, $P < 0.01$). [Reproduced with permission from P. A. Beranek *et al.*: *Int J Cancer* 36:685–687 © 1985 Wiley-Liss, Inc. (56) (panel A) and Y. Chin *et al.*: *Anticancer Res* 12:1463–1466, 1992 (134) (panel B).]

VIII. Regulation of Lymphocyte Cytokine Production

A. Th1 and Th2-helper cells

There is now good evidence for a role of macrophages and lymphocytes producing cytokines that may stimulate breast tumor estrogen synthesis. It is therefore important to consider what regulates cytokine production by these cells. While little is known about the regulation of macrophage cytokine production, important advances have been made in the regulation of lymphocyte cytokine production (Fig. 6). It is now evident that T helper (Th) cells, a type of lymphocyte, can exist as two distinct subsets (135, 136). Th cells can mature to either a Th1 or Th2 phenotype, each of which secretes

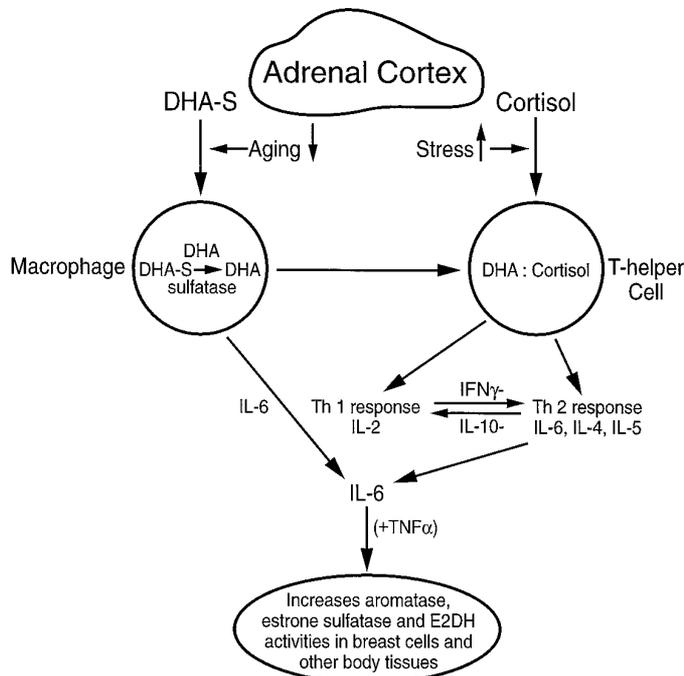


FIG. 6. The role of the endocrine system in regulating the formation of Th1 or Th2 cells. T helper (Th) cells can mature to either a Th1 or Th2 phenotype, each of which secretes a characteristic profile of cytokines. Th1 cells secrete IL-2 and $\text{IFN}\gamma$ whereas Th2 cells secrete IL-4, IL-5, IL-6, and IL-10. IL-6 can stimulate estrogen synthesis in breast cancer cells and can act synergistically with $\text{TNF}\alpha$ to enhance enzyme activities. The response of Th1 and Th2 cells is mutually exclusive with $\text{IFN}\gamma$ inhibiting the formation of Th2 cells and IL-10 the formation of Th1 cells. A major factor regulating the progression of Th cells to either the Th1 or Th2 phenotype is the balance of the adrenal androgen, DHA, to that of the glucocorticoid, cortisol. Within the lymphoid tissue environment, where maturation of Th cells occurs, DHA-sulfatase, which is present in macrophages, has a crucial role in regulating the availability of DHA from DHA-sulfate (DHA-S). Aging is associated with a decrease in plasma DHA/DHA-S concentration, and production of these adrenal androgens is decreased in women with breast cancer, favoring a Th2 cytokine response with increased secretion of IL-6, which can stimulate tumor estrogen synthesis. Stress can act to increase glucocorticoid production and therefore also provokes a Th2 cytokine response and thus increased IL-6 secretion and estrogen synthesis. [Adapted from M. J. Reed *et al.*: *J Steroid Biochem Mol Biol* 53:413–420 (127). © 1995 with kind permission from Elsevier Science Ltd., The Boulevard, Langford Lane, Kidlington OX5 1GB, UK.]

a characteristic set of cytokines. For example, Th1 cells secrete $\text{IFN}\gamma$, IL-2, and $\text{TNF}\beta$, while Th2 cells secrete IL-4, IL-5, IL-6, and IL-10. Cytokines produced by Th2 cells therefore include IL-6 which, as previously discussed, is emerging as having an important role in regulating breast tumor estrogen synthesis. Furthermore the responses of Th1 and Th2 cells are mutually inhibitory. $\text{IFN}\gamma$, which is secreted by Th1 cells, inhibits secretion of cytokines by Th2 cells, while IL-10, which is secreted by Th2 cells, inhibits the Th1 cytokine response.

B. Dehydroepiandrosterone (DHA) and glucocorticoids

Important clues as to what regulates the production of Th1 or Th2 cytokines have recently been provided by the studies of Daynes *et al.* (137, 138) and Rook *et al.* (139). Plasma IL-6

concentrations were found to be elevated in elderly human subjects (140, 141) but also in aged mice (141). In aged mice, however, it was possible to correct the elevated IL-6 levels *in vivo* by the acute or chronic administration of DHA or DHA-sulfate (DHA-S) (141). These studies have revealed that *in vitro* DHA, but not DHA-S, is able to suppress the release of Th2 cytokines, indicating that DHA sulfatase, which is present in macrophages within the lymphoid tissues where Th cell maturation occurs and which converts DHA-S to DHA, has a crucial role in regulating part of the immune response. From such studies it emerged that the balance of DHA to glucocorticoids is what governs whether T cells progress to develop a Th1 or Th2 phenotype and the secretion of different types of cytokines. *In vivo* and *in vitro* DHA is now known to possess potent antiglucocorticoid properties. Therefore, in conditions associated with decreased DHA or DHA-S production, as occurs in aging (142), T cells will progress to Th2 cells with a concomitant increase in IL-6 production. Stress, which is associated with an increase in glucocorticoid production, will also alter the balance between DHA and glucocorticoids and favor a Th2-type cytokine response. Thus, an increase in the production of Th2-type cytokines, which includes IL-6, will result in a coordinated increase in the three enzymes involved in estrogen synthesis in peripheral and normal and malignant breast tissues. The presence of specific DHA receptors on murine and human T cells has now been reported (143, 144).

IX. Clinical Observations Explained by Proposed Model of Cytokine Regulation of Estrogen Synthesis

A. Septic shock

Although the role that cytokines have in regulating estrogen synthesis in peripheral tissues, including the breast, is actively being investigated, it was, in fact, first suggested some years ago that factors involved in regulating estrogen synthesis may result from the body's response to injury or infection (145). Striking evidence in support of this concept was provided in 1988 by Nunez and his colleagues, who showed that plasma estrogen concentrations were markedly elevated in male septic shock patients. Plasma estrone levels were increased 13-fold and estradiol concentration 5-fold in shock subjects compared with normal subjects (146) (Fig. 7). It is now known that plasma IL-6 and $\text{TNF}\alpha$ concentrations are elevated in patients with septic shock, and the observation by Nunez therefore provides important evidence in support of a role for these cytokines in regulating peripheral estrogen synthesis (147, 148).

B. The effect of aging on peripheral estrogen synthesis

With the realization that IL-6 has a central role in regulating peripheral aromatase activity, it becomes possible to suggest a mechanism to account for the increase that has been detected, as a result of aging, from both *in vivo* (25) and *in vitro* (26, 28) studies. As previously discussed, it is now thought that the decrease in the production of the adrenal androgens DHA and DHA-S that occurs with aging results in an increase in production of the Th2-type cytokines, which

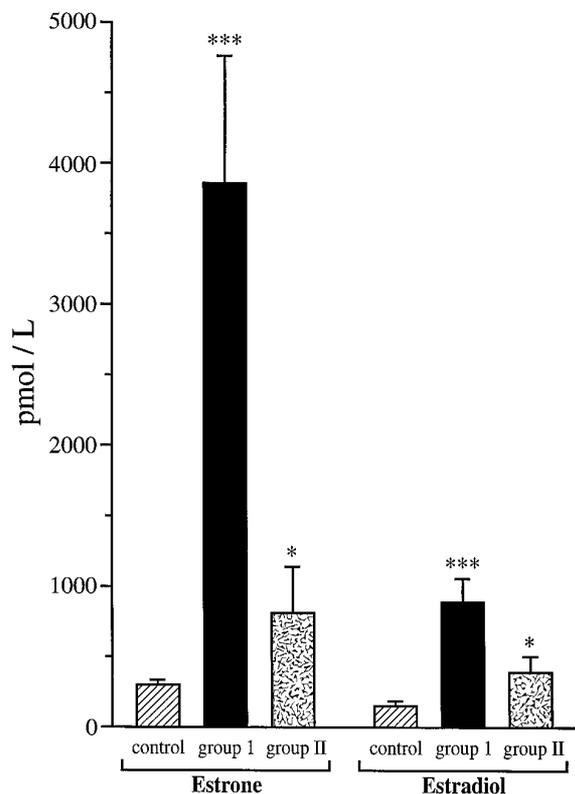


FIG. 7. Plasma estrone and estradiol concentrations in men with septic shock who subsequently died (group I) or recovered (group II). Plasma IL-6 (119) and TNF α (120) concentrations are increased in subjects with septic shock, and this most likely accounts for the high plasma estrogen levels detected in these subjects. [Reproduced from N. Christeff *et al.*: *J Steroid Biochem Mol Biol* 29:435–440 (146). © 1988 with kind permission from Elsevier Science Ltd., The Boulevard, Langford Lane, Kidlington OX5 1 GB, UK.]

includes IL-6. Increased production of IL-6 by Th2 cells, and its effect on aromatase activity, therefore offers a likely explanation for the age-related increase that occurs for peripheral estrone synthesis.

C. The effect of weight on peripheral aromatase activity

TNF α , which stimulates aromatase, E2DH, and estrone sulfatase can act, as previously discussed, synergistically with IL-6 to increase the activities of these enzymes. In addition to being produced by cells of the immune system, TNF α is also secreted by adipocytes (91, 92). However, a greater amount of TNF α is secreted by adipocytes from obese subjects than by those of lean individuals (149). Thus, although the increased mass of adipose tissue, in which aromatization takes place, of obese subjects is likely to contribute to their increased production of estrone (3), it is likely to be potentiated by their increased production of TNF α from adipocytes.

D. The discriminant function test

Some years ago Bulbrook and Hayward developed the discriminant function test as a marker for women who subsequently developed breast cancer (150). The excretion of low

levels of androgen metabolites of DHA indicated women at risk of developing breast cancer and were also associated with an unfavorable prognosis in women with breast cancer. These studies were extended to show that the discriminant function test, *i.e.*, the ratio of 11-deoxy-17-oxosteroids (mainly etiocholanolone and androsterone, which are derived from adrenal DHA) to that of 17-hydroxyglucocorticoids (largely derived from cortisol) improved the predictive power of such hormone measurements. While it was not readily apparent 30 yr ago why the test had such predictive value, it was recently postulated (151) that the discriminant function test may, in fact, be a marker of Th1/Th2 cytokine production. Adrenal androgen production is reduced in women with breast cancer, and thus an imbalance in the DHA-glucocorticoid ratio would favor the production of Th2 cytokines including IL-6. Increased production of IL-6 would result in an increase in estrogen synthesis in peripheral and breast tissues. There is also some evidence that in addition to T lymphocytes, malignant cells themselves might also secrete Th2-type cytokines (152).

E. Stress and breast cancer

The role of stress in increasing the risk of breast cancer is controversial, but a recent study showed convincing evidence for a higher number of life-stress events in women with breast cancer but not benign breast disease (153). Although the way in which stress influences the development of breast cancer is very complex (154), stress, as previously discussed, would be expected to increase glucocorticoid production. As breast cancer occurs most commonly in postmenopausal women, a time when DHA/DHA-S production is in decline, then any increase in stress would move the T-helper cell response in the direction of Th2 cytokine production and could result in increased estrogen synthesis. BALB/c mice, subjected to water stress after Meth A tumor transplantation, have an increase in their tumor size and tumor growth rates compared with unstressed animals (155).

F. Immunosuppression and breast cancer risk

The incidence of breast cancer in kidney transplant recipients receiving immunosuppressive therapy is 25% lower than expected in a normal population (156). Immunosuppressive drugs, such as cyclosporin A, act to inhibit white blood cell (wbc) production and the secretion of cytokines by these cells. Therefore in immunosuppressed women reduced amounts of cytokines would be available to stimulate estrogen synthesis in breast and other peripheral tissues and may contribute to the reduced incidence of breast cancer in these patients.

Support for the use of immunosuppressive drugs leading to a reduction in wbc cytokine production and estrogen synthesis has been obtained (69). Collection of CM from wbc of an immunosuppressed subject revealed a marked reduction in its cytokine content and its ability to stimulate aromatase activity compared with CM collected from a woman with breast cancer.

G. Failure of glucocorticoids to stimulate aromatase activity *in vivo*

If IL-6 is an important factor regulating *in vivo* aromatase activity, then this might offer some explanation for the discrepancy, as previously noted, in the ability of glucocorticoids to stimulate *in vitro* but not *in vivo* aromatase activity (30, 33, 34). Dexamethasone can only induce aromatase activity *in vitro* in the presence of FCS, but the reason for this and the factors facilitating the ability of dexamethasone to stimulate aromatase activity are not known. A factor with a molecular mass in the region of 150–300 kDa has been isolated in FCS as being responsible for stimulating aromatase activity, but its identity is not yet known (157). As dexamethasone induces IL-6R expression, it is possible that it acts *in vitro* to increase the number of IL-6Rs on cells (158), thereby increasing the ability of IL-6, which may be present in FCS in some complexed form, to stimulate aromatase activity. However, while glucocorticoids act to increase IL-6R expression, they act *in vivo* to inhibit IL-6 gene expression, possibly as part of a feedback mechanism to limit the response to stress and infection (159). Inhibition of IL-6 gene expression by glucocorticoids *in vivo* could therefore account for their inability to stimulate *in vivo* aromatase activity.

X. Summary and Future Perspectives

The results from the research reviewed have led to a clearer understanding of the complexities of the regulation of estrogen synthesis in breast tumors and provided a possible explanation for the effects of aging and body weight on peripheral estrogen synthesis and stress on the increased risk of breast cancer. It is evident that cytokines, such as IL-6 and TNF α , have important roles in stimulating estrogen synthesis in breast cancer cells although it is likely that other stimulatory factors remain to be identified. Cells of the immune system, which are attracted to infiltrate tumors by chemokines, are probably the major source of cytokines and cytokine-soluble receptors that have been found to stimulate estrogen synthesis. However, IL-6 derived from stromal tissue and TNF α from adipocytes within the breast are also likely to contribute to the production of regulatory cytokines.

A major paradox that has emerged from much of the research carried out during the last few years to isolate factors that stimulate estrogen synthesis is the realization that most factors that stimulate enzyme activity *in vitro* also inhibit cell growth (160). The only possible exception to these findings was for IGF-I/II, which while stimulating E2DH activity, also tended to increase cell growth (81). A possible explanation for this paradox may lie with the fact that macrophages and lymphocytes, which probably produce most of the cytokines that stimulate estrogen synthesis, are trying to act to inhibit the growth of tumor cells, *i.e.*, carrying out their immunosurveillance role. However, *in vivo* with the availability of the appropriate substrates, *i.e.*, androstenedione, estrone, and estrone sulfate, the stimulation of the activities of the enzymes that utilize these substrates produces sufficient estradiol to overcome the normal inhibitory effect that cytokines have on cell growth. Also, it is possible that the production of IL-6 sR by tumor fibroblasts and/or invading

macrophages and lymphocytes overcomes the normal desensitization that would be expected to occur if these cells were producing large amounts of IL-6. Thus, in addition to oncogene activation, which can result in overexpression or aberrant functioning of growth factors and their receptors, it is possible that IL-6 sR production by cells that are in close proximity to malignant cells is yet another mechanism whereby normal cell growth mechanisms are subverted, thus enabling tumor cells to proliferate.

The concept that increased production of estrogens enables tumor cells to overcome the inhibitory effects of the immune system is in keeping with the hypothesis first put forward some years ago by Prehn and Lappé (161). They postulated that the immune response was unlikely to have evolved for the purpose of promoting tumor growth and speculated "that the effects of the immune system on tumor growth must be the inadvertent consequences of some attributes of the response that are more advantageous to the individual." It also remains a possibility, however, that in addition to IL-6 and TNF α , which inhibit *in vitro* cell growth, other cytokines may not only stimulate estrogen synthesis but also cell growth. Leukemia-inhibitory factor, for example, which stimulates aromatase activity (95), is known to stimulate the proliferation of MCF-7 breast cancer cells (162) and to be produced by ER– but not ER+ breast cancer cells (163). It is also possible that another cytokine, IL-3, which has been shown to be secreted by fibroblast derived from male breast tissue but not female and which can inhibit E2DH reductive activity (164), may also have a role in the complex regulation of breast tumor estrogen synthesis.

The last 10 yr has been an exciting period for research into the regulation of breast tumor estrogen synthesis. Not only have the enzymes involved in estrogen synthesis been isolated and their genes cloned (13–19) but specific inhibitors for some of the enzymes have also been developed (165–168). However, it is becoming evident that the use of enzyme inhibitors alone may not be sufficient to produce the anticipated clinical benefits of total estrogen synthesis blockage (169, 170). At some stage in the near future it will therefore be necessary to develop novel strategies for inhibiting the growth of hormone-dependent breast tumors. Investigations to reveal the complex signaling pathway (95) whereby cytokines control enzyme activity may lead to the development of specific inhibitors of the signal transduction pathways that lead to increased estrogen synthesis in tumors. Understanding the interaction of cytokines with their receptors (171), and the factors that regulate their production and expression, could lead to the development of cytokine receptor antagonists that could have a therapeutic role in the treatment of breast cancer.

If, as discussed in this review, DHA or a related metabolite, such as androstenediol or androstenediol (172, 173), does have an important antiglucocorticoid role in regulating the Th1/Th1 balance, it should be possible to obtain further evidence of an imbalance in cytokine production and thus a role for this steroid in the etiology of breast cancer. Also, the possibility of using DHA or DHA-S for the chemoprevention of breast cancer would appear to warrant further investigation.

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