Introduction

The use of aromatase inhibitors to prevent breast cancer represents only an hypothesis at the present time. Substantial evidence in model systems and from clinical observations supports the plausibility of this hypothesis. In this treatise, we will review evidence suggesting that estrogens can cause breast cancer, that estradiol is synthesized in breast tissue via the enzyme aromatase, that estrogens can be metabolized to genotoxic metabolites, and that aromatase overexpression is associated with breast dysplasia and cancer in rodent models and in an aromatase over-expressing transgenic mouse model. These data will be used to support the concept that trials of aromatase inhibitors for prevention of breast cancer are warranted at the present time.
Evidence that estrogens cause breast cancer

Administration of exogenous estrogens causes breast cancer in rodents (Furth 1982). Development of breast tumors by the potent carcinogens, dimethylbenzanthracine (DMBA) and nitrosomethylurea, can be delayed or prevented by castration and administration of antiestrogens (Hollingsworth et al. 1998). Aromatase inhibitors prevent the spontaneous development of benign and malignant breast lesions in aging Sprague-Dawley rats (Gunson et al. 1995). Nearly 50% of these animals develop breast lesions, half of which are malignant, by the age of 2 years. Increasing doses of the aromatase inhibitor, fadrozole, inhibit these tumors in a dose-dependent fashion. The 1.25 mg/kg dose causes nearly complete prevention of lesions in all animals studied. Taken together, these data provide strong evidence that estrogens are involved in the carcinogenic process resulting in breast neoplasms in animals.

Evidence in women also supports a role for estrogen in causing breast cancer. Early menarche and late menopause are associated with an increased risk of breast cancer. These factors result in prolonged exposure of the breasts to estrogen. Recent prospective epidemiologic studies demonstrated a 20% increase in the plasma levels of estradiol in women who were found to develop breast cancer 5 or more years later (Wysowski et al. 1987, Toniolo et al. 1995, Berrino et al. 1996, Dorgan et al. 1997). Obesity is also associated with a greater risk of breast cancer (Magnusson et al. 1998). This relationship might also be explained by increased estrogen production since the degree of obesity correlates linearly with total body aromatase activity (Longcope et al. 1986). Aromatase is an enzyme which catalyzes the rate-limiting step in estrogen biosynthesis, the conversion of androgens to estrogens.

Additional evidence regarding estrogen and breast cancer derives from analyses of the rate of breast cancer in women receiving estrogen replacement therapy during the menopause. More than 50 studies are now available to examine this relationship and six meta-analyses have pooled this information in order to draw conclusions from larger data bases (Collaborative Group on Hormonal Factors in Breast Cancer 1997). Most studies show an increased risk of breast cancer with long-term use of estrogen replacement therapy; generally for a period of more than 5-10 years. The relative risk of breast cancer under these circumstances increases by about 30%.

Further evidence regarding estrogen and breast cancer risk has been provided by experiments demonstrating that a reduction of estrogen production in women reduces the incidence of breast cancer. Data from two classic studies are consistent with such an effect (Figs 1 and 2) (Feinleib 1968, Trichopoulos et al. 1972). One of these studies examined the incidence of breast cancer in a group of women who had undergone bilateral oophorectomy before the age of 35. The control group consisted of women subjected to a unilateral oophorectomy at the same ages. The endpoint of the study was the ratio between observed and expected breast cancers in these two groups of women. After a period of 20 years, the women undergoing bilateral oophorectomy had a 75% reduction in the incidence of breast carcinoma (Fig. 1). In a second study, with a similar design, the decrease in breast cancer incidence over that expected gradually declined as a function of time after oophorectomy (Fig. 2). Although these studies were also subject to bias, they provide compelling evidence that ovarian factors, and presumably estradiol, are involved in the genesis of breast carcinoma.
Physiology of estradiol production

Concentrations of the enzyme aromatase and of its substrates, testosterone and androstenedione, provide the primary determinants of estradiol production. The sources of estrogen synthesis in women are important to consider since overproduction may result from altered regulation at any site. Estrogen can be made in several tissues since aromatase, the primary regulator of the level of production of estradiol, is widely present throughout the body. The premenopausal ovary contains the highest level of aromatase and is the major source of estradiol during the premenopausal years. Levels of androstenedione, the substrate for aromatase in the ovary, are also substantial and regulated by the level of luteinizing hormone (LH) secreted by the pituitary. Peripheral adipose tissue also contains large amounts of aromatase since the mass of adipose tissue, particularly in obese women, is substantial. More recently recognized is the fact that breast tissue itself contains aromatase and can synthesize estrogen in situ.

Importance of in situ aromatase in breast tissue

Emerging evidence suggests the importance of estrogen production in situ in the breast. Several experimental systems have been used to determine the magnitude and biological importance of in situ estrogen production by breast tissue. These include demonstration of the aromatase enzyme and its mRNA in breast tissue by immunohistochemical and molecular biological techniques, studies in nude mice to show that the amount of estrogen made locally causes biological effects, and clinical studies of aromatase inhibitors in patients.

Several groups of investigators over the past several years utilized radiometric and product isolation aromatase assays to demonstrate the presence of aromatase in breast cancer (Bezwoda et al. 1987, Miller & O’Neill 1987, Lipton et al. 1992). Immunohistochemical data demonstrated high focal levels of aromatase staining and supported the concept that aromatase might act in an autocrine or paracrine fashion in breast tissue (Estaban et al. 1992, Santen et al. 1994, Berstein et al. 1996).

Controversy exists at present as to whether aromatase activity is predominantly in epithelial cancer cells or in the surrounding stromal cells. Isolated stromal cells from breast cancer tissue contain high levels of aromatase enzyme which can be stimulated by nearly four orders of magnitude by dexamethasone, phorbol esters and cyclic AMP in combination (Santer et al. 1997). In addition, aromatase message can be stimulated by the same cocktail of enhancers in this tissue. Finally, levels of aromatase message are higher in areas of breast cancer with high stromal cell content than in areas with low content (Bulun et al. 1996). Taken together, these data support the biological importance of aromatase in breast cancer tissue and suggest that stroma may contribute to a greater extent to this process than epithelial cells.

Further support for the importance of aromatase in breast tissue itself derives from studies in the nude mouse model. These recently published experiments examined the relative importance of uptake from plasma versus local estradiol synthesis (Yue et al. 1998). MCF-7 breast cancer cells transfected stably with aromatase were implanted on one side of castrated nude mice. On the other side, sham-transfected MCF-7 cells were implanted. Administration of the aromatase substrate androstenedione caused no growth stimulation of aromatase-negative cells. This important control demonstrated that no biologically significant aromatase activity is present in non-breast tissue. Aromatase-positive cells implanted on the other side of the same animals were stimulated to grow by androstenedione, providing evidence of the biological effect of aromatase present locally in breast. The aromatase inhibitor, 4-hydroxy (OH) androstenedione blocked this growth effect. As expected, the levels of estradiol in aromatase-positive tumors markedly exceeded those in aromatase-negative tumors.

The relative importance of in situ production versus uptake of estradiol from plasma was then examined. Silastic implants designed to produce plasma estrogen levels ranging from 5 to 20 pg/ml were implanted into castrate animals to evaluate the effect of estradiol uptake. Androstenedione was administered to others to examine in situ production. With this experimental system, tissue estradiol levels and tumor growth were higher as a result of in situ aromatization than from plasma delivery of estrogen.

This series of experiments has led to the hypothesis that an important determinant of tissue estradiol levels is local production in the breast. If this hypothesis is correct, it is the level of estradiol in tissue which is the most important determinant of estradiol-induced carcinogenesis. This fact may explain the relatively poor, albeit statistically significant, correlation between plasma estrogen levels and breast cancer development (Wysowski et al. 1987, Toniolo et al. 1995, Berrino et al. 1996, Dorgan et al. 1997). Following this line of reasoning, the concentration of estradiol in breast tissue itself would be a more precise predictor of later development of breast cancer.

Mechanism of carcinogenesis

After considering the sources of estradiol available for stimulating breast tissue, one must consider how estradiol causes breast cancer. Enhanced cell proliferation, induced either by endogenous or exogenous estrogens, increases the number of cell divisions and, by inference, the
proportionate number of mutations (Preston-Martin et al. 1993). With an enhanced rate of proliferation, the time available for DNA repair is reduced. Additionally, single-stranded DNA, present during cell division, is more susceptible to damage than double-stranded and gene duplication can occur. This concept of estrogen-induced carcinogenesis represents the predominant thinking at the present time. Recently, a number of investigators have focused on another carcinogenic mechanism, the metabolism of estrogens to genotoxic products (Fig. 3). It is postulated that estradiol is metabolized to 4-OH estradiol and then to estrogen quinones. The quinones bind to guanine and are removed from DNA through a glycosidase enzyme to form N-3-adenine-quinone or N-7-guanine-quinone conjugates (Fig. 3). The remaining segment of depurinated DNA forms G to T and A to T point mutations upon replication. In addition, the quinones can be reduced back to 4-OH estradiol through the activity of quinone reductase. 4-OH estradiol and 3,4 estradiol quinone can then participate in a redox cycle with generation of oxygen-free radicals with each revolution through the cycle. These free radicals can directly damage DNA. It is plausible to consider that these two mechanisms, increased cell proliferation and genotoxic effects of estradiol metabolites, act together in either a synergistic or additive fashion (Fig. 4).

A critique of the concept of the genotoxic effect of estradiol metabolites is that estrogen levels are not sufficiently high to produce biologically relevant amounts of these metabolites. This critique, however, is based upon an analysis of plasma estradiol levels and not tissue levels. If estradiol is synthesized locally in breast tissue, the levels would be higher than expected from plasma concentrations. This concept is supported by the fact that estradiol concentrations in breast cancers from postmenopausal women are as high as those from

Figure 3 Biochemical pathways in which estradiol is metabolized to genotoxic metabolites. Estradiol and estrone can be metabolized to either 2- or 4-OH estradiol via specific P450 enzymes. The 2-OH compounds can cause stable DNA adducts. The 4-OH compounds are metabolized to 3,4 quinones which can then bind covalently to guanine or adenine and cause depurinating adducts. The catechol-o-methyl-transferase (COMT) pathway and the glutathione pathway detoxify the catecholestrogens by forming methylated derivative such as 4-methoxy estradiol or glutathione conjugates.
premenopausal women (Van Landeghem et al. 1998). This is surprising, since the levels of estradiol in the plasma of premenopausal women are 10- to 50-fold higher than in postmenopausal women.

**Hypothesis of aromatase overexpression**

As a means of integrating these data, we have postulated that aromatase overexpression in breast tissue may be a cause of breast cancer. Through aromatase overexpression, tissue levels of estradiol would be sufficiently high to undergo metabolism to biologically important quantities of genotoxic metabolites. Four separate models of aromatase overexpression and breast cancer have been well characterized and provide strong support for our hypothesis. Three involve the hyperplastic alveolar nodule (HAN) model systems. Dr Daniel Medina has developed a series of transplantable breast explants which grow in the mammary fat pads of highly inbred strains of mice (Zhang & Medina 1993). Two of these are induced by carcinogens and are called the C4 and C5 HANs. One is induced by hormonal stimulation and is called the D2 HAN. Upon serial passage in mammary fat pads, these lesions develop frank cancer with an incidence which approaches 90% under certain conditions. Each of these HAN models has been shown to have an insertional mutation, called Int 5. This mutation has now been characterized and involves the insertion of a portion of the long terminal repeat of the mouse mammary tumor virus into genomic DNA (Tekmal & Durgam 1995). Of great interest is the fact that, in each of these models, the insertion is into the 3'-untranslated region of the tenth exon of the aromatase gene. This results in overexpression of the aromatase gene and, by inference, in tumor development. The fourth model is a transgenic mouse in which aromatase is overexpressed predominantly in mammary tissue. These animals develop typical and atypical ductal hyperplasia, fibroadenomas, and dysplasia. Such lesions, when diagnosed in patients, are known to be associated with a high risk of development of breast cancer (Tekmal et al. 1996).

**Evidence of aromatase in benign breast tissue**

We have obtained evidence that benign breast tissue contains aromatase message and enzyme activity. Evaluation of breast biopsies containing atypical ductal hyperplasia revealed aromatase immunohistochemical staining in both stromal and epithelial cells (Mor et al. 1998). In the normal tissue elements surrounding these lesions, the aromatase staining was present in glandular epithelial cells. In the lesions themselves, stromal staining was present.

We also detected macrophages with substantial aromatase activity. To demonstrate that macrophages indeed contain aromatase activity, we conducted a series of experiments using THP-1 cells, a malignant cell line which can be differentiated into macrophages upon exposure to phorbol esters (Mor et al. 1998). These cells contain aromatase enzyme activity with levels close to those found in human placenta. The aromatase inhibitor letrozole completely inhibited this activity. Conditioned media from these cells, given the aromatase substrate testosterone, stimulated the growth of estradiol-responsive MCF-7 indicator cells. As evidence of specificity, growth of indicator cells could be blocked with letrozole or with the pure antiestrogen ICI 782,982.

Human monocytes grown in culture differentiated into macrophages with addition of phorbol esters. These cells contained aromatase message when differentiated into macrophages but not under control conditions. Finally,
monoclonal antibodies specific for macrophages were used to demonstrate by double labeling that the cells in the breast which contained aromatase were in fact macrophages. These data suggest that breast tissue can make estradiol from epithelial cells, from stroma, and from macrophages which infiltrate normal tissue. Potentially, any one of these three cell types could overexpress aromatase and provide sufficient amounts of estradiol locally to allow conversion to genotoxic quinone metabolites.

Evidence of aromatase overexpression

Several examples of aromatase overexpression are known to exist. A rat Leydig-cell tumor overexpresses aromatase through activation of a cyclic AMP-dependent enhancer of aromatase (Fitzpatrick & Richards 1994). The breast tissue of goats is the major source of aromatase prior to parturition and bilateral mastectomy delays the time of parturition (Peaker & Taylor 1990). The Sebright-Bantam syndrome is caused by aromatase overexpression which feminizes the feather pattern of roosters and gives them the phenotypic appearance of chickens (Wilson et al. 1987). Familial causes of aromatase overexpression occur in patients and result in prepubertal gynecomastia in boys and precocious thelarche and/or macromastia in girls (Simpson et al. 1997). The Peutz-Jeagher syndrome results in aromatase overproducing testicular tumors in boys and ovarian tumors in girls (Coen et al. 1991).

Mechanism of aromatase overexpression

A variety of potential mechanisms could produce aromatase overexpression. Aromatase transcription is regulated by a wide variety of enhancers, including cyclic AMP, phorbol esters, dexamethasone, prostaglandin E₂, transforming growth factor-β, and γ-interferon among others (Simpson et al. 1997). We have demonstrated that fibroblasts isolated from breast tumors as well as from benign tissue surrounding the tumors contain aromatase. The activity of this enzyme and its message can be stimulated up to 10,000-fold in cell culture with addition of phorbol esters, cyclic AMP, and dexamethasone (Santner et al. 1997). Activating mutations involving any of these or other steps could result in aromatase overexpression in breast tissue. Simpson et al. (1997) have postulated that prostaglandin E₂ may be important in this process and have pointed out that use of non-steroidal anti-inflammatory agents (NSAIDs) is associated with a decreased incidence of breast cancer in women (Zhao et al. 1996). The NSAIDs are known to block prostaglandin E₂ production and putatively could decrease breast cancer through this mechanism.

Aromatase inhibitors in breast cancer prevention

The concepts presented above provide a rational framework for the hypothesis that aromatase inhibitors might be used to prevent breast cancer. Potent and highly specific third-generation inhibitors are available that could now enter clinical trials as preventative agents. These trials are now warranted based upon several considerations. The aromatase inhibitor, fadrozole, has been shown to prevent spontaneous breast cancer in rodents (Gunson et al. 1995). The antiestrogens, tamoxifen and raloxifene, reduced the onset of new breast cancers by 45% and 71% respectively when administered to women for a period of 30 months to 4 years (Cummings et al. 1998, Wickerham et al. 1998). These data support the feasibility of preventing breast cancer by hormonal means. Long-term administration of aromatase inhibitors in women with breast cancer has shown them to be well tolerated and safe. However, accelerated bone loss and/or cardiovascular disease could result from a profound lowering of estradiol. Nonetheless, it appears appropriate to initiate trials of aromatase inhibitors in women selected appropriately.

Potential advantages of aromatase inhibitors over antiestrogens for breast cancer prevention

Antiestrogens block estradiol-induced cell proliferation but not the synthesis of estradiol. If the genotoxic effects of estradiol metabolites are important, aromatase inhibitors would block their formation in addition to decreasing the rate of cellular proliferation. By lowering the levels of tissue estradiol, smaller amounts of substrate would be available to be converted into 4-OH estradiol; 3,4 estradiol quinone, and into guanine or adenine conjugates. Theoretically then, the aromatase inhibitors might be more effective in preventing the initiation of breast cancer than the antiestrogens. Both would be expected to reduce the tumor-promotion step by counteracting the effects of estrogen on cellular proliferation.

It should be recognized that biological information regarding the reduced incidence of breast cancer associated with antiestrogen administration is incomplete. It is possible that these agents act merely as chemosuppressants and do not decrease the ultimate incidence of breast cancer. Pre-existing, preclinical, hormone-dependent breast cancers would be expected to stop growing or regress in response to tamoxifen or raloxifene. This would result in a decreased incidence of newly detected breast cancer after a period of 30 months to 4 years (the duration of the National Surgical Adjuvant Breast Project (NSABP) study; Wickerham et al. 1998).
Two other studies, albeit smaller, have not confirmed the tumor-preventative effect of tamoxifen (Powles et al. 1998, Veronesi et al. 1998). In a commentary regarding these studies, Dr Kathleen Pritchard suggested that long-term use of tamoxifen might result in its becoming an estrogen agonist (Pritchard 1998). If correct, short- but not long-term use of tamoxifen would result in chemosuppression of tumors.

Another observation in the NSABP prevention trial also favors the concept of chemosuppression. If tamoxifen were inhibiting the growth of pre-existing tumors, one would expect that the cancers which did occur would be nearly exclusively estrogen receptor negative and thus hormone independent. Both the raloxifene and tamoxifen trials did find that the majority of tumors detected were estrogen receptor negative. While not firm evidence, these observations are consistent with the chemosuppression hypothesis.

Knowledge of the mechanistic basis for estrogen-induced carcinogenesis is critical to the rationale for use of aromatase inhibitors for breast cancer prevention. If the genomic, cell proliferation pathway acts in an additive or synergistic fashion with the genotoxic pathway, use of aromatase inhibitors could truly prevent breast cancer. Both estrogen receptor-positive and estrogen receptor-negative cancers would be prevented. These concepts will require much additional experimental evidence before conclusive proof is obtained. Trials of aromatase inhibitors in women at high risk for breast cancer might be a practical means to test these hypotheses while awaiting more conclusive experimental proof of the underlying pathophysiology.

**Aromatase inhibitors in pre- and postmenopausal women**

The premenopausal ovary is relatively resistant to blockade with aromatase inhibitors (Santen et al. 1980). This results from the dual effects of high tissue levels of androstenedione as substrate in the ovary and the reflex rise in LH and follicle-stimulating hormone (FSH) which occur when estradiol levels are lowered. As a consequence, LH stimulates further production of androstenedione by the theca cells of the ovary and FSH induces synthesis of increased amounts of aromatase. These combined effects allow the ovary to continue producing significant amounts of estradiol.

How then could aromatase inhibitors prevent breast cancer in premenopausal women? This depends upon the primary source of breast tissue estradiol levels. If local aromatization in breast is important, aromatase inhibitors would block this in situ synthesis. In breast tissue, aromatase is not subject to regulation by FSH and tissue levels of androstenedione as substrate are much lower than in the ovary. Support for the concept of in situ synthesis rather than uptake of plasma estradiol derives from the nude mouse experiments cited above (Yue et al. 1998). This hypothesis, while not proven, is attractive since it suggests that one can lower breast tissue estradiol levels without causing a reduction in plasma estradiol levels. This would protect a patient from development of osteoporosis, urogenital atrophy, and vasomotor instability while still reducing the incidence of breast cancer.

Aromatase inhibitors in postmenopausal women would also lower levels of tissue estradiol. No reflex effects on negative feedback would be expected. The plasma levels of estradiol would also fall as has been shown in women being treated for breast cancer. The consequences on potential acceleration of osteoporosis, urogenital atrophy, and on vasomotor instability would have to be carefully assessed. These issues have not been addressed in substantial detail in women with advanced breast cancer because of the relatively short duration of exposure to aromatase inhibitors in them.

**Conclusion**

Metabolism of estradiol to genotoxic quinone metabolites provides a rational hypothesis to explain the mammary carcinogenic effects of estrogen in women. The plausibility of this hypothesis has been questioned on the grounds that insufficient estradiol is present in breast tissue to be converted to biologically significant amounts of metabolite. This critique is based upon measurement of plasma estradiol levels and the assumption of concordance between plasma and tissue estradiol levels. However, this assumption is clearly not correct since estradiol levels in postmenopausal breast tumors are similar to those in premenopausal women. Plasma estradiol levels are 10- to 50-fold higher in pre- than in postmenopausal women. Consequently, factors must be present to alter breast tissue estradiol levels independently of plasma concentrations. One such factor may be the local production of estradiol in breast tissue through the enzyme aromatase. If correct, mutations or environmental factors enhancing aromatase activity would result in high tissue concentrations of estradiol. This concept, if verified experimentally, would provide plausibility to the hypothesis that sufficient estradiol may be present in tissue for genotoxic metabolites to be important. This line of reasoning provides a strong rationale for use of aromatase inhibitors to prevent breast cancer and for the potential superiority of aromatase inhibitors over use of antiestrogens for this purpose.

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