Endocrine-Related Cancer (1999) **6** 307-314

Aromatase overexpression and breast hyperplasia, an *in vivo* model – continued overexpression of aromatase is sufficient to maintain hyperplasia without circulating estrogens, and aromatase inhibitors abrogate these preneoplastic changes in mammary glands

R R Tekmal[1], N Kirma[1], K Gill[1] and K Fowler[2]

[1]Department of Gynecology and Obstetrics, Emory University School of Medicine, 4219 WMB, 1639 Pierce Drive, Atlanta, Georgia 30322-4710, USA

[2]Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia, USA

(Requests for offprints should be addressed to R R Tekmal)

Abstract

To test directly the role of breast-tissue estrogen in initiation of breast cancer, we have developed the aromatase-transgenic mouse model and demonstrated for the first time that increased mammary estrogens resulting from the overexpression of aromatase in mammary glands lead to the induction of various preneoplastic and neoplastic changes that are similar to early breast cancer. Continued overexpression of aromatase that leads to increased breast-tissue estrogen contributes to a number of epigenetic changes in mammary tissue such as alteration in the regulation of genes involved in apoptosis, activation of genes involved in cell cycle and cell proliferation, and activation of a number of growth factors. Our current studies show aromatase overexpression is sufficient to induce and maintain early preneoplastic and neoplastic changes in female mice without circulating ovarian estrogen. Preneoplastic and neoplastic changes induced in mammary glands as a result of aromatase overexpression can be completely abrogated with the administration of the aromatase inhibitor, letrozole. Consistent with complete reduction in hyperplasia, we have also seen downregulation of estrogen receptor and a decrease in cell proliferation markers, suggesting aromatase-induced hyperplasia can be treated with aromatase inhibitors. Our studies demonstrate that aromatase overexpression alone, without circulating estrogen, is responsible for the induction of breast hyperplasia and these changes can be abrogated using aromatase inhibitors.

Endocrine-Related Cancer (1999) **6** 307-314

Introduction

Breast cancer is one of the most prevalent types of cancer observed in women. The mitogenic and proliferative effects of estrogens have long been recognized and are known to correlate with estrogen and progesterone receptors in breast tumor tissue. Interestingly, the proportion of patients with hormone-sensitive tumors is greater among postmenopausal patients than premenopausal patients (McGuire 1980, Lippman & Dickson 1989). In addition, the source of sex steroids differs between pre- and postmenopausal women. Previous studies have demonstrated the presence of aromatase, the rate-limiting enzyme responsible for estrogen biosynthesis in breast tumors (Miller & Forrest 1974, Siiteri 1982, Santner *et al.* 1984, Reed *et al.* 1989). Breast tumors from postmenopausal women maintain a high estrogen content, even though the plasma concentrations of

1351-0088/99/006-307

estradiol decrease to low values after menopause. One pathway for *in situ* synthesis involves the conversion of androstenedione to estrone/estradiol, catalyzed by aromatase. Since aromatase was first detected in breast tumors, the problem of assessing its functional significance has attracted considerable attention and controversy. A number of recent studies provide evidence to support a biological role for tumor aromatase (see review by Tekmal & Santen 1998).

An intriguing hypothesis is that local estrogen is directly involved in the initiation of either preneoplastic or neoplastic (or both) changes in mammary epithelium. To address this question directly, we have generated an aromatase-transgenic mice model and showed for the first time that the transgenic virgin and postlactational females that overexpress aromatase develop various preneoplastic histopathological changes (Tekmal *et al.* 1996). These observations led us to the current studies, which examined the importance of circulating estrogen, if any, in maintaining the preneoplastic and neoplastic changes that were induced in breast tissue as a result of aromatase overexpression. In addition, we wished to demonstrate that preneoplastic/neoplastic changes induced as a result of aromatase overexpression can be completely abrogated with the administration of aromatase inhibitors. Accordingly, we examined the continued growth and maintenance of aromatase-induced hyperplasia in the absence of circulating ovarian estrogens and the ability of aromatase inhibitors such as letrozole to completely eliminate or reduce the aromatase induced breast hyperplasia using our *in vivo* model.

Materials and Methods

Aromatase-overexpressing transgenic mice and animal treatments

The generation of aromatase-transgenic mice (*MMTV-int-5/aromatase*) and their characterization have been described previously (Tekmal *et al.* 1996). Briefly, aromatase cDNA was expressed under the control of tissue-specific mouse mammary tumor virus promoter (MMTV). Aromatase-transgenic mice overexpressing aromatase were maintained in a centralized, fully accredited animal facility.

Effect of ovariectomy on breast hyperplasia in aromatase-transgenic mice

To determine the influence of circulating ovarian estrogens, if any, on aromatase-induced breast hyperplasia in transgenic mice, we ovariectomized both virgin and postlactational transgenic mice along with non-transgenic littermates and implanted them with androstenedione pellets (each pellet provides 100 µg/animal per day) as a steady source of substrate for aromatase. Mammary glands from both control and aromatase-transgenic mice were examined 2 months after ovariectomy and examined for any histopathological changes.

Treatment with aromatase inhibitor

Aged (about 12 months old) virgin aromatase-transgenic females were used to investigate the effect of aromatase inhibitor, letrozole, as a chemopreventive agent to eliminate or reduce the preneoplastic and neoplastic changes induced in mammary glands of these mice. In this study, about 1-year-old virgin, age-matched transgenic females were divided into two groups: one group ($n=6$) served as control group, the other group received daily s.c. injection of letrozole (5 µg letrozole/animal in 100 µl 0.3% hydroxypropyl cellulose in phospate buffered saline). Control animals were given s.c. injection of vehicle. Letrozole was a gift from Drs Ajay Bhatnagar and Dean Evans of Novartis Pharma (Basel, Switzerland). At the end of 6 weeks of treatment, mammary glands were removed and one gland from each animal was used for histological analysis and all other glands were pooled and used for biochemical analysis as described below.

Mammary glands from both ovariectomized and letrozole-treated animals, along with glands from control animals, were removed and processed for histological examination as described before (Tekmal *et al.* 1996).

Biochemical analysis of mammary tissues

Total RNA from mammary tissues of aromatase-transgenic mice and control non-transgenic littermates was isolated as described before (Chomczynski & Sacchi 1987). Equal amounts of total RNA from both control transgenic animals and transgenic animals treated with letrozole were analyzed for estrogen receptor (ER) mRNA levels by RT-PCR as described before (Hou & Gorski 1993). To demonstrate that an equal amount of total RNA was used from each sample to determine ER expression, we examined the expression of *GAPDH*, a housekeeping gene, as an invariant control by RT-PCR. The densitometric data from ethidium bromide staining of RT-PCR products on agarose gels were used for calculating the differences in the expression of ER mRNA levels in various samples.

Mammary tissues from both control and letrozole-treated animals were also used to determine the levels of proliferating cell nuclear antigen (PCNA) using western blot analysis. Briefly, mammary tissue from both control and letrozole-treated animals was homogenized in lysis buffer and 60 µg total protein from each sample was separated on polyacrylamide gels and transferred to nylon membrane. Non-specific binding antibodies were blocked by incubation for at least 1 h at room temperature with

Tris-buffered saline containing 0.05% Triton X-100 (TBST) and 5% non-fat dry milk. Filters were exposed to antibodies (PCNA or actin, an invariant housekeeping protein control) in TBST-milk for 1 h at room temperature, and specific binding was visualized by using antimouse IgG followed by enhanced chemiluminescent detection (ECL kit; Amersham). The densitometric data from western blots (X-ray image of chemiluminescent proteins) were used for calculating the differences in the expression of PCNA levels in various samples.

Results

Our previous studies (Tekmal *et al.* 1996, Gill *et al.* 1998) clearly indicated that overexpression of aromatase leads to increased estrogenic activity both in female and male mammary glands and in male reproductive tissues, which results in the initiation of various preneoplastic/neoplastic changes in female and male mammary glands and Leydig cell tumor formation in males. To determine whether circulating ovarian estrogens has any role in the persistent growth of these preneoplastic and neoplastic changes induced by aromatase overexpression in mammary glands of transgenic animals, we ovariectomized postlactational aromatase-transgenic females along with non-transgenic littermate controls. Mammary glands from these animals were examined 2 months after ovariectomy for persistence of aromatase-induced preneoplastic and neoplastic changes. Data presented in Fig. 1 clearly show that the preneoplastic and neoplastic changes persist, even in the absence of ovarian estrogens in ovariectomized animals. These results clearly demonstrate that overexpression of aromatase that results in increased mammary estrogen alone is sufficient to induce/maintain various pre-neoplastic/neoplastic changes in the mammary gland, without the influence of circulating ovarian estrogens.

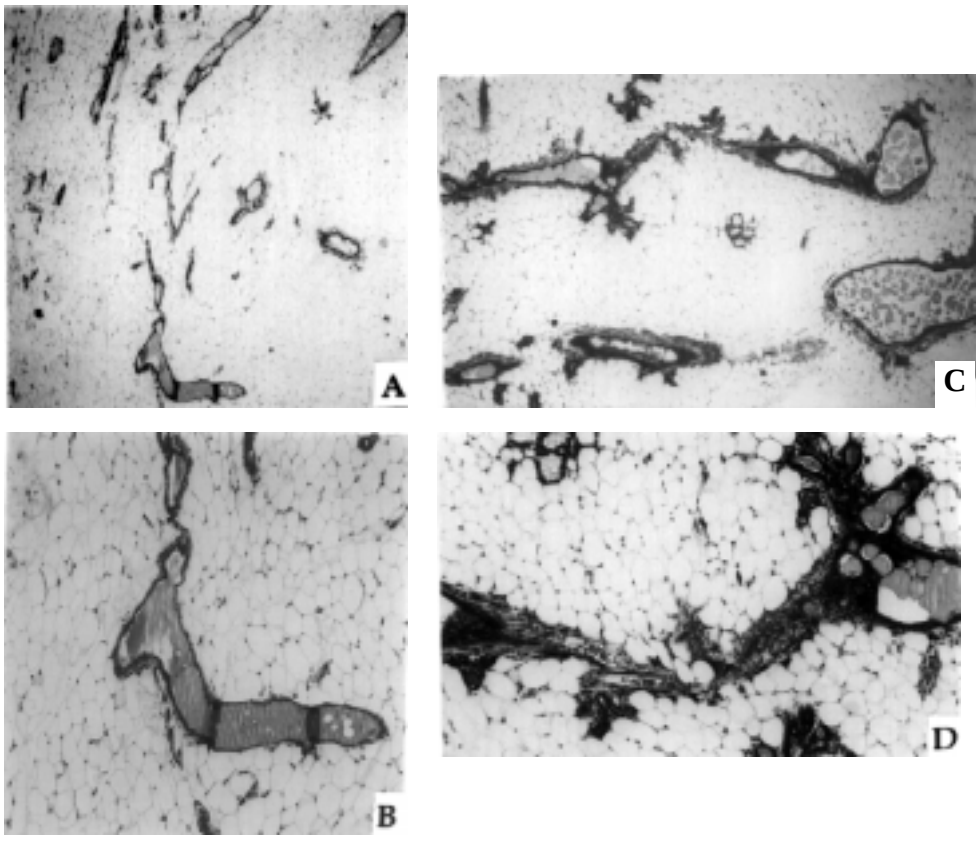

Figure 1 Histological sections of mammary glands from postlactational ovariectomized mice. A and B show that there are few ducts and only rudimentary glandular epithelial growth in mammary glands of non-transgenic mice. Magnifications: A, ×12.5; B, ×25. C and D show continued hyperplastic and dysplastic glandular and hyperplastic ductal growth in mammary glands of aromatase-transgenic mice. Magnifications: C, D ×25.

To test whether the preneoplastic/neoplastic changes induced by aromatase in transgenic mice can be completely eliminated or reduced by aromatase inhibitors, we have treated aromatase-transgenic females with the aromatase inhibitor, letrozole, to completely abrogate or reduce aromatase-induced hyperplastic and other changes in breast tissue. Mammary glands from these animals were examined after completion of 6 weeks of letrozole treatment. Data presented in Fig. 2 clearly show that, compared with control aromatase-transgenic females, the aromatase females treated with letrozole for 6 weeks show complete reduction/disappearance of hyperplasia and other preneoplastic and neoplastic changes that were induced by the overexpression of aromatase in these animals. These results clearly demonstrate that breast hyperplasia and other preneoplastic/neoplastic changes that were induced by aromatase overexpression can be abrogated by potent aromatase inhibitors such as letrozole.

To determine whether letrozole also influenced other endocrinological effects in ovary and uterus, we examined both circulating concentrations of estradiol and histological changes, both in uterus and in ovaries of letrozole-treated animals, in comparison with control animals. Our results show that, compared with mean circulating concentrations of estradiol (11.61 pg/ml) in control aromatase-transgenic females, the concentrations of estradiol in letrozole-treated females were very low - below the sensitivity of detection using a conventional assay (Table 1). Histological examination (data not shown) of both uterus and ovaries from letrozole-treated animals in comparison with control animals show typical effects of estrogen deprivation. These results suggest that

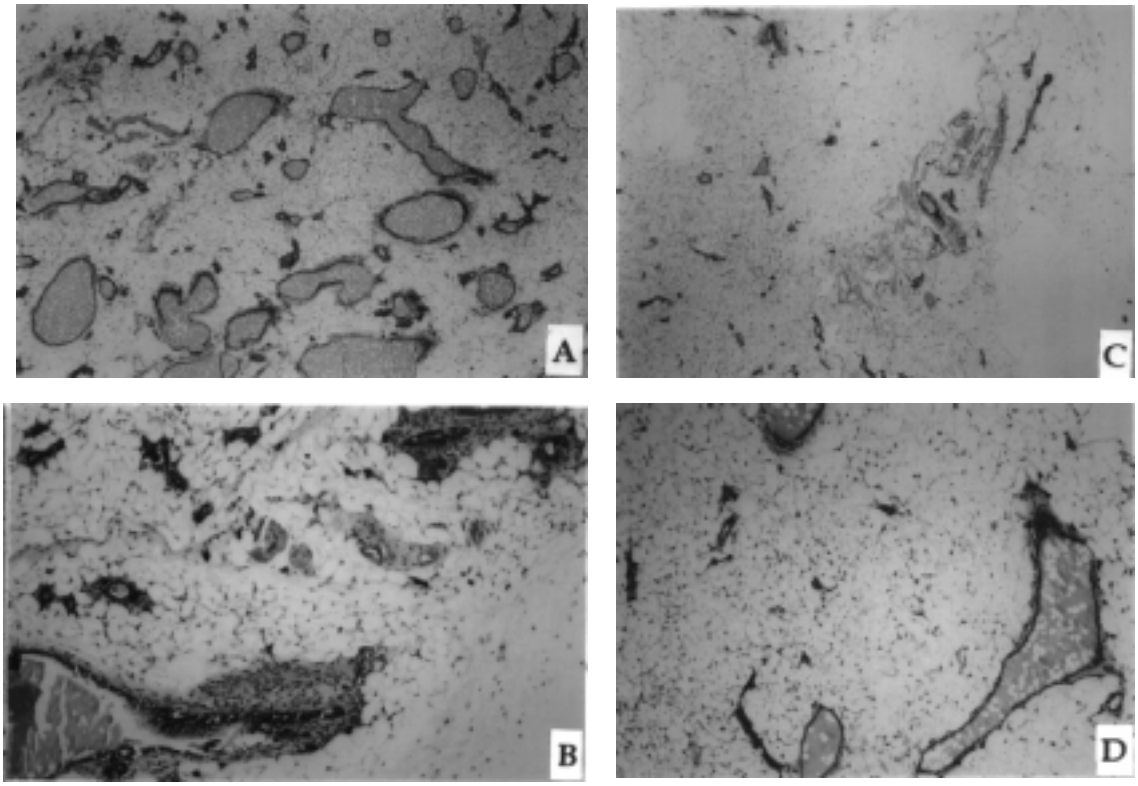

Figure 2 Effect of letrozole on aromatase-overexpression-induced breast hyperplasia in aromatase-transgenic female mice. A, B: Histological sections of mammary gland from aromatase-transgenic female mice. Magnifications: A, ×12.5; B, ×25. C, D: Histological sections of mammary gland from letrozole-treated aromatase-transgenic female mice. Magnifications: C, ×12.5; D, ×25. Note the abrogation of glandular and ductal hyperplasia and the drastic decrease in number of ducts and ductal enlargement in letrozole-treated animals.

Table 1 Circulating estradiol concentrations in control and letrozole-treated aromatase-transgenic mice

Serial No.	Experimental group (pg/ml)*	Control group (pg/ml)*
1	1.74	11.31
2	2.91	10.89
3	2.04	11.92
4	2.60	12.37
5	1.13	11.58
Mean value	2.084±0.70	11.614±0.566

Estradiol concentrations were estimated using a commercial assay kit (Diagnostic Products Corp., Los Angeles, CA, USA) and duplicate samples.
* The sensitivity of the assay was 5.0 pg/ml, on the basis of a 100 µl sample size.

the dose of letrozole (5 µg/animal/day) we used not only led to complete reduction/elimination of breast hyperplasia and other preneoplastic and neoplastic changes, but also affected other normal endocrine functions.

To determine whether blocking of aromatase leads to a decrease in cell proliferation as a result of lack of continuous mitogenic stimulation from tissue estrogen, we estimated the levels of PCNA in mammary tissues from letrozole-treated animals in comparison with control transgenic mice. The data presented in Fig. 3 clearly show that the mammary tissue PCNA protein level in letrozole-treated animals was about tenfold lower than that of control animals. These results are consistent with histological observations, which showed a marked decrease or complete elimination of breast hyperplasia in letrozole-treated animals.

Our previous studies have shown that overexpression of aromatase in transgenic mammary glands leads to upregulation of estrogen receptor (Kirma *et al.* 1998), in addition to induction of various histopathological changes in mammary epithelial cells (Tekmal *et al.* 1996). Therefore, we have examined whether reduction in the mammary hyperplasia as a result of letrozole treatment also leads to downregulation of estrogen receptors. Our results show (Fig. 4) that, compared with ER mRNA levels in mammary tissue of control animals, the levels of ER mRNA in mammary tissue of letrozole-treated animals were almost tenfold lower. These results suggest that blocking of estrogen by letrozole leads to down-regulation of ER in mammary tissue.

Discussion

Since aromatase was first detected in breast tumors (Miller & Forrest 1974), the problem of assessing its functional

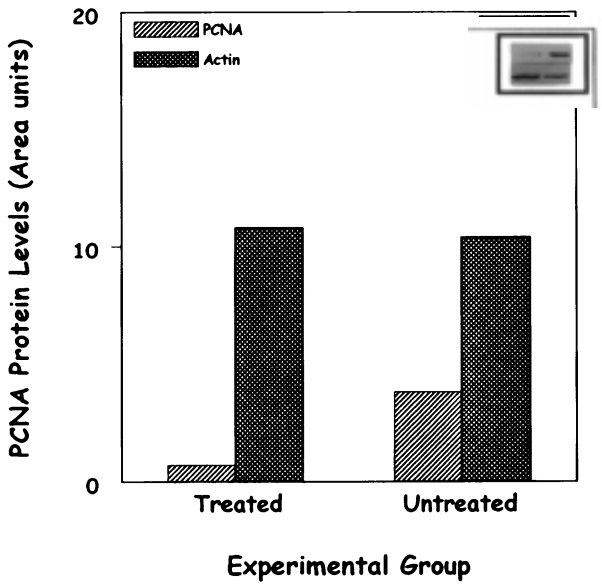

Figure 3 Expression of PCNA in mammary gland tissues of control and letrozole-treated aromatase-transgenic female mice. Inset: Equal amounts of total protein from control and letrozole-treated mammary glands were analyzed by western blot. Densitometric data from western blot analysis were used for graphic representation.

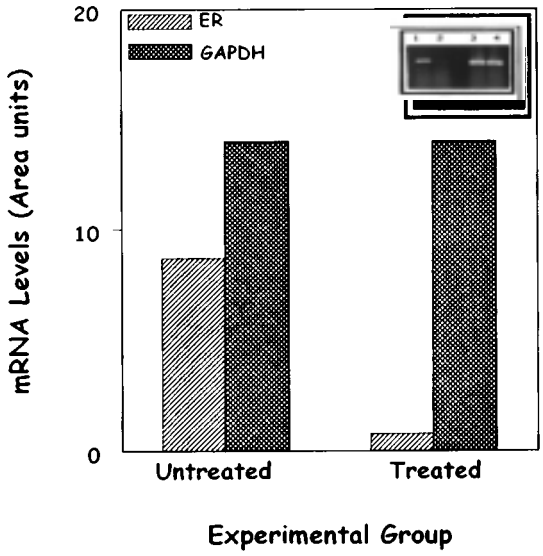

Figure 4 Expression of ERα mRNA in mammary gland tissues of control and letrozole-treated aromatase-transgenic female mice. Inset: Equal amounts of total RNA (500 ng) from control (lane 1) and letrozole-treated (lane 2) aromatase-transgenic female mammary gland tissues were analyzed by RT-PCR using ERα primers. Lanes 3, 4: RT-PCR amplification of *GAPDH* as an invariant control, shown to demonstrate the equal amount of RNA in each sample. Densitometric data from ethidium bromide fluorescence of RT-PCR products on agarose gel were used for graphic representation.

significance has attracted considerable attention and controversy. A number of recent studies have provided evidence to support a biological role for tumor aromatase (Evans *et al.* 1993, Brodie & Santen 1994, Reed 1994). Aromatase activity and aromatase mRNA are higher in tumor-bearing quadrants than in normal quadrants (O'Neil *et al.* 1988, Bulun *et al.* 1996). Tumor concentrations of androstenedione are known to be greater than plasma concentrations and contribute to significant *in situ* tumor estrogen synthesis (Miller & Forrest 1976, Perel *et al.* 1980, Santner *et al.* 1984). Increased aromatase protein expression in tumors compared with normal areas of the breast was indicated by immunohistochemical localization (Lu *et al.* 1996). Breast tumor-specific transcriptional regulation of aromatase gene (Harada *et al.* 1993, Chen 1998). In addition, breast tumor aromatase activity can be correlated with markers of cell proliferation such as PCNA (Lu *et al.* 1996). All these studies provide evidence that locally overexpressed aromatase has a direct biological role in the growth of breast tumors.

An intriguing hypothesis is that local estrogen is directly involved in the initiation of either preneoplastic or neoplastic (or both) changes in mammary epithelium. To address this question directly, we have generated an aromatase-overexpression transgenic model (previously referred to as *int-5/ aromatase* transgenic mousce model). Our initial studies using this model (Tekmal *et al.* 1996, Gill *et al.* 1998) showed that overexpression of aromatase leads to increased estrogenic activity both in female and male mammary glands and in male reproductive tissues, which results in the initiation of various preneoplastic/ neoplastic changes in female and male mammary glands and Leydig cell tumor formation in males. We have also shown (Kirma *et al.* 1998) that overexpression of aromatase leads to various epigenetic changes that may be involved in estrogen-induced tumorigenesis.

The data presented here suggest that aromatase overexpression leads to induction of breast hyperplasia and other preneoplastic/neoplastic changes, and that these changes persist even without any circulating estrogen in ovariectomized animals. Our recent studies (Gill *et al.* 1998) showed that overexpression of aromatase in male transgenic mice results in the increased epithelial glandular growth as early as 10 weeks of age. Histological examination of these glands reveals clear evidence of hyperplastic and dysplastic changes that are similar to gynecomastia (a benign condition). Furthermore,

aromatase overexpression in male reproductive tissues alters the hormonal milieu in these tissues and leads to the induction of Leydig cell tumors in these animals. These data, taken together, clearly demonstrate that increased tissue estrogen alone, without the influence of circulating estrogen, is sufficient to induce various preneoplastic/ neoplastic changes.

A large body of literature provides direct evidence that aromatase inhibitors are very effective in blocking the action of this enzyme and in treating hormone-dependent breast tumors (Brodie & Santen 1994, Brodie & Njar 1998). We wanted, therefore, to investigate whether aromatase inhibitors can be used as chemopreventive agents to block/reduce aromatase-induced breast hyperplasia and other preneoplastic/neoplastic changes in transgenic animals. Data presented here clearly demonstrate that the aromatase inhibitor, letrozole, used in our experiments is very effective in abrogating aromatase-induced breast hyperplasia. However, the dose we used in our initial studies, based on those used in previous animal studies (Brodie *et al.* 1998), not only abrogated breast hyperplasia but also affected some normal endocrinological functions in these animals. Nevertheless, these results do provide clear evidence that these inhibitors are very effective in blocking aromatase-induced breast hyperplasia. However, more studies are needed to determine the minimum dose of aromatase inhibitor required to potentially block the enzyme activity in tissues such as breast but not in ovary, and will shed more light on the efficacy of aromatase inhibitors as potential chemopreventive agents to block the initiation and progression of estrogen-dependent breast cancers.

Recent studies by Brodie *et al.* (1997) showed that the presence of high levels of aromatase in tumor epithelial cells correlated with increased cell proliferation as shown by increased PCNA levels in these cells. Our recent studies (Gill *et al.* 1998, Kirma *et al.* 1998) also showed a clear correlation between increased PCNA levels and breast hyperplasia and gynecomastia in female and male transgenic mice respectively. These studies suggest that locally produced estrogen may play a role in stimulating proliferation. If this is indeed the case, decreased aromatase levels should also result in a decrease in cell proliferation and a decrease in PCNA levels. Our data are in complete agreement with this hypothesis.

A number of previous studies (Silva *et al.* 1989, Miller *et al.* 1990, Brodie *et al.* 1997) showed no consistent relationship between ER status and tumor aromatase levels. However, a majority of ER-positive tumors had a high aromatase activity. We have recently shown that overexpression of aromatase led to upregulation of ERα in mammary tissue of transgenic females and also activation of ERα in transgenic male mammary glands (Gill *et al.* 1998, Kirma *et al.* 1998). Data presented here clearly showed complete downregulation of ERα in mammary tissues of animals that were treated with aromatase inhibitor, suggesting that lack of breast-tissue estrogen led to downregulation of its receptors. We have not only seen upregulation of ERα (Kirma *et al.* 1998) in aromatase overexpressing transgenic mammary glands, but also activation of ERβ in these tissues (N Kirma & R R Tekmal, unpublished observations).

Our studies suggest that overexpression of aromatase that results in increased breast-tissue estrogen initiates a number epigenetic changes through its receptor-mediated mechanism, which lead to the induction of breast hyperplasia and other preneoplastic/neoplastic changes.

Acknowledgements

This work was supported by a grant from NIH/NCI CA 75018. The authors thank Jessica Mantione and Mamata Parida for technical assistance.

References

Brodie AMH & Santen RJ 1994 Aromatase and its inhibitors in breast cancer - overview and perspective. *Breast Cancer Research and Treatment* **30** 1-6.

Brodie AM & Njar VC 1998 Aromatase inhibitors in advanced breast cancer: mechanism of action and clinical implications. *Journal of Steroid Biochemistry and Molecular Biology* **66** 1-10.

Brodie A, Lu Q & Nakamura J 1997 Aromatase in the normal breast and breast cancer. *Journal of Steroid Biochemistry and Molecular Biology* **61** 281-286.

Brodie A, Lu Q, Liu Y, Long B, Wang JP & Yue W 1998 Preclinical studies using the intratumoral aromatase model for postmenopausal breast cancer. *Oncology* **12** 36-40.

Bulun SE, Sharda G, Rink J, Sharma S & Simpson ER 1996 Distribution of aromatase P450 transcripts and adipose fibroblasts in the human breast. *Journal of Clinical Endocrinology and Metabolism* **81** 1273-1277.

Chen S 1998 Aromatase and breast cancer. *Frontiers in Bioscience* **3** d922-d933.

Chomczynski P & Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162** 156-159.

Evans TRJ, Rowlands MG, Silva MC, Law M & Coombes RC 1993 Prognostic significance of aromatase and estrone sulfatase enzymes in human breast cancer. *Journal of Steroid Biochemistry and Molecular Biology* **44** 583-587.

Gill K, Keshava N, Mantione J & Tekmal RR 1998 Overexpression of int-5/aromatase in transgenic male mice leads to gynecomastia and testicular cancer [Abstract, No. 3751]. *Proceedings of the American Association for Cancer Research* **39** 551.

Harada N, Utsumi T & Takagi Y 1993 Tissue-specific expression of the human aromatase P-450 gene by alternative use of multiple exons 1 and promoters, and switching of tissue-

specific exons in carcinogenesis. *Proceedings of the National Academy of Sciences of the USA* **90** 11312-11316.

Hou Q & Gorski J 1993 Estrogen receptor and progesterone receptor genes are expressed differentially in mouse embryo during preimplantation development. *Proceedings of the National Academy of Sciences of the USA* **90** 9460-9464.

Kirma N, Keshava N, Gill K, Fang G, Bhalla KN & Tekmal RR 1999 Aromatase overexpression in mammary glands of transgenic mice results in increased tissue estrogen and leads to breast hyperplasia and the induction number of epigenetic changes. *Cancer Research* (In Press).

Lippman ME & Dickson RB 1989 Mechanisms of growth control in normal and malignant breast epithelium. *Recent Progress in Hormone Research* **45** 383-440.

Lu Q, Nakamura J, Savinov A, Yue W, Weisz J, Dabbs D, Wolz J & Brodie A 1996 Expression of aromatase protein and mRNA in tumor epithelial cells and evidence of functional significance of locally produced estrogen in human breast cancers. *Endocrinology* **137** 3061-3068.

McGuire WL 1980 An update on estrogen and progesterone receptors in prognosis for primary and advanced breast cancer. In *Hormones and Cancer,* pp 337-344. Eds S Iacobelli *et al.* New York: Raven Press.

Miller WR & Forrest APM 1974 Oestradiol synthesis by a human breast carcinoma. *Lancet* **ii** 866-868.

Miller WR & Forrest APM 1976 Oestradiol synthesis from C19 steroids by human breast cancers. *British Journal of Cancer* **33** 116-121.

Miller WR, Anderson TJ & Jack WLJ 1990 Relationship between tumor aromatase activity, tumor characteristics and response to therapy. *Journal of Steroid Biochemistry and Molecular Biology* **37** 1055-1059.

O'Neill JS, Elton RA & Miller WR 1988 Aromatase activity in adipose tissue from breast quadrants: a link with tumor site. *British Medical Journal* **296** 741-743.

Perel E, Wilkins D & Killinger DW 1980 The conversion of androstenedione to estrone and estradiol, and testosterone in breast tissue. *Journal of Steroid Biochemistry and Molecular Biology* **13** 89-92.

Reed MJ 1994 The role of aromatase in breast tumors. *Breast Cancer Research and Treatment* **30** 7-17.

Reed MJ, Owen AM, Lai LC, Coldham NG, Ghilchik MW, Shaikh NA & James VHT 1989 *In situ* oestrone synthesis in normal breast and breast tumor tissues: effect of treatment with 4-hydroxyandrostenedione. *International Journal of Cancer* **44** 233-237.

Santner SJ, Feil PD & Santen RJ 1984 *In situ* estrogen production via the estrone sulfatase pathways in breast tumors: relative importance versus the aromatase pathway. *Journal of Clinical Endocrinology and Metabolism* **48** 29-33.

Siiteri PK 1982 Review of studies on estrogen biosynthesis in the human. *Cancer Research* **42** 3269S–3275S.

Silva MC, Rowlands MG, Dowsett MD, Gusterson B, McKinna JA, Fryatt I & Coombes RC 1989 Intratumoral aromatase as a prognostic factor in human breast carcinoma. *Cancer Research* **49** 2588-2591.

Tekmal RR & Santen RJ 1999 Local estrogen production: is aromatase an oncogene? In *Contemporary Endocrinology: Endocrinology of the Breast*. Ed A Manni. Totowa, NJ: Humana Press (In Press).

Tekmal RR, Ramachandra N, Gubba S, Durgam VR, Mantione J, Toda K, Shizuta Y & Dillehay DL 1996. Overexpression of *int-5/aromatase* in mammary glands of transgenic mice results in the induction of hyperplasia and nuclear abnormalities. *Cancer Research* **56** 3180-3185.