Normal Breast Tissue Implanted into Athymic Nude Mice Identifies Biomarkers of the Effects of Human Pregnancy Levels of Estrogen

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Abstract We have generated a novel model system for the study of estrogen intervention in normal breast tissue. Nulliparous human breast tissue was implanted into immunocompromised nude mice and treated with high-dose estrogen to simulate the effects of pregnancy. Treatment of mice with human mid-pregnancy levels of 17β-estradiol for a period of 4 weeks was followed by 4 weeks of withdrawal to mimic involution. Gene expression in the xenograft tissue was then analyzed by real-time reverse transcription-PCR to identify differences between treated and control tissues. Ten genes previously identified as altered by pregnancy in rodent models were found to be differentially expressed in human breast tissue with a ≥1.8-fold up-regulation of CDC42, TGFβ3, DCN, KRT14, LTF, and AREG and a ≥0.7-fold down-regulation of STAT1, CTGF, IGF1, and VAMP1. Immunohistochemical analysis of archival paraffin-embedded adult premenopausal human breast tissue specimens identified a significantly lower level of expression of STAT1 (P < 0.05, Mann-Whitney U test) in parous compared with age-matched nulliparous tissue (median of 24% compared with 42% epithelial cells positive). We conclude that many of the pregnancy-induced breast cancer–protective changes observed in rodent models also occur in human breast tissue following intervention using human pregnancy levels of estrogen and that STAT1 expression is a potential biomarker of parity-induced breast cancer protection in the human breast.

A late first full-term pregnancy (FFTP) has been shown to increase the risk of breast cancer, whereas an early FFTP is known to be highly protective (1). Women who have their FFTP before the age of 18 have one third the breast cancer risk of those whose FFTP is delayed until age 35 or older (2). This protective effect of pregnancy can be reproduced in animal models where an early FFTP has been shown to confer resistance to the subsequent induction of tumors by chemical carcinogens (3). Mimicking the protective effects of pregnancy can be achieved in rodents by treatment with pregnancy levels of 17β-estradiol (E2) and progesterone, although treatment with E2 alone was shown to be sufficient to reduce susceptibility to mammary carcinogenesis (4). It was found that the period of hormone treatment need not be that of full gestation (3 weeks), with a treatment period of only 1 week being sufficient to significantly reduce the incidence of mammary carcinogenesis (4). The mechanisms for this protective effect have not been fully elucidated although several hypotheses have been proposed to explain the effect including systemic changes in endocrine hormones, mammary differentiation, or other changes in the mammary epithelium and stroma.

Parity results in persistent alteration of the hormonal environment in the mammary gland. It has been found that parity in rats results in significant persistent reductions in circulating levels of growth hormone and also in mammary gland levels of both estrogen receptor and epidermal growth factor receptor (5). In humans, it has been shown that parous women have significantly lower levels of serum prolactin when compared with nulliparous women, but there is no association with age at first birth (6). Levels of prolactin in postmenopausal women are also known to be associated with mammographic density, which is itself is strongly correlated to a high risk of breast cancer (7). Changes in the responsiveness of the gland to hormonal stimulation have been proposed as a possible mechanism for parity-induced protection. In part, the changes in response to hormonal stimulation may be due to alterations in the stromal component of the gland. A recent report showed that transplantation of transgenic p53-null epithelium into the cleared mammary fat pad of hosts previously treated with E2 and progesterone was as effective in preventing tumors as hormonal treatment of the p53-null mouse itself (8).
 Alternatively, the protective effect of parity may result from an increased state of differentiation and a reduction in proliferation within the mammary gland. It has been suggested that the protective effect is due to the complete differentiation in the mammary gland (9), although this is not supported by a study showing that near full lactational differentiation induced by increasing circulating prolactin levels in the absence of pregnancy does not protect against carcinogenic challenge (10). Several studies have identified molecular changes in parous mammary tissue compared with that from nulliparous animals. In rats, one group found that mimicking pregnancy through hormone treatment results in up-regulation of genes identified as markers of mammary differentiation, metabolism, extracellular matrix (ECM), cell contact, and regulatory factors including signaling molecules and transcription factors (11). Another group using microarray analysis of murine mammary glands showed that parity resulted in the persistent down-regulation of genes encoding for growth factors and up-regulation of genes encoding for the inhibitory growth factor transforming growth factor β3 (TGFβ3) and several of its downstream targets. In addition, there was up-regulation of several markers of differentiation and genes involved with inflammatory response (12). A core signature of 70 differentially regulated, parity-induced genes were identified that are conserved across several rat strains. This signature included up-regulation of differentiation markers and immune response genes, whereas there was reduction in expression of growth factor–related genes and genes of the growth hormone/insulin-like growth factor axis (13). The lists of parity-induced genes from these studies are largely nonoverlapping.

The fact that the protective effects of pregnancy can be reproduced by a relatively short period of E2 treatment offers the potential for developing new strategies for preventing breast cancer in humans. We have successfully used a mouse xenograft model to investigate the effect of serum concentrations of estradiol equivalent to those observed in the luteal phase of the menstrual cycle on normal human breast cell proliferation (14–16) and gene expression (17). Here, we develop a xenograft mouse model of human breast tissue where the effects of estrogen intervention can be tested by implanting nulliparous human tissue and treating with high-dose E2 pellets to achieve human pregnancy levels of serum E2 for a 1-month period.

Materials and Methods

Human Tissues

Histologically normal human female breast tissues were obtained from 14 nulliparous women (mean age, 24 ± 2.4 y) undergoing operation for removal of benign lesions or reduction mammoplasty with the approval of South Manchester Research Ethics Committee. After obtaining patient consent, full details of reproductive histories were recorded. Tissue was collected and stored in DMEM (Life Technologies, Inc.) for a maximum period of 4 h until processed for mouse implantation or histology. In addition, an anonymized collection of age-matched paraffin-embedded archival specimens of normal breast tissue collected from both nulliparous (n = 15; mean age, 34 y) and parous (n = 15; mean age, 34 y) premenopausal women together with the appropriate details of their reproductive history was available (18). All samples used in the parous group came from women whose first full-term pregnancy occurred before the age of 25.

Animals

Animals used for the xenograft experiments were 6- to 10-wk-old female athymic nude BALB/c nu/nu mice from Harlan Ltd., whereas those for measurement of serum hormone levels were female BALB/c nu/nu littermates of the same age. All surgical procedures were done in accordance with the Animals (Scientific Procedures) Act 1986 and under isoflurane inhalation anesthesia (3-5% isoflurane, 50-70% O2/NO2 at 2-4 L/min; Fluvox Vapouriser, International Market Supplies). Subsequent to the surgical procedures, all animals received treatment with analgesic (Rimadyl, 0.1 mL/20 g of body weight).

Assessment of serum hormone levels

To measure serum levels of E2, female BALB/c nu/nu mice were assigned to one of three groups of 24 animals. Animals in each group received an implant of silastic pellets containing 6, 12, or 18 mg of E2, whereas control animals received sham pellets. All pellets were inserted s.c. on the dorsal surface close to the tail, and sites of incision were sealed with surgical wound clips. Four animals per group were anesthetized and exsanguinated via cardiac puncture at 14-d intervals to a total time of 84 d. To investigate the effects of the E2 implants on levels of endogenous murine prolactin and growth hormone, three groups of eight female BALB/c nu/nu mice each received silastic implants of one of the three doses of estradiol as described above. These were removed after 28 d, at which point four animals from each dosing regimen were exsanguinated, with the remaining animals being sacrificed at 56 d. Any mice showing signs of E2 toxicity during the course of any experiment were immediately sacrificed and excluded from any further analysis. E2 toxicity was judged to be present by the appearance of red sores on the skin and problems with passing urine due to hypertrophy of the urogenital tract. The collected blood was centrifuged at 2,000 rpm for 10 min at 4°C, and serum was stored at −70°C for subsequent analysis of hormone levels done on a Bayer Immuno-One assay analyzer.

Simulating human pregnancy in a mouse xenograft model

Each normal human breast tissue sample (n = 14) was stripped of excess fat under sterile conditions and divided into pieces of ∼2 × 1 × 1 mm. A total of eight mice were used per human sample (control, n = 2; treated, n = 6). Two small incisions were made across the midline dorsal skin through which eight tissue pieces were symmetrically placed as described previously (14). Fourteen days after xenograft implantation, implants equivalent to 6, 12, or 18 mg of E2 were inserted as described above. These were maintained in the mice for a period of 28 d at which point the pellets were excised by sharp dissection. Control animals received the same treatment but using an equivalent number of sham pellets for each dosing regimen. The timing of xenograft harvest is illustrated in Fig. 1. Two xenografts were excised at 14, 21, 42, and 70 d after tissue implantation. At day 70, all animals were sacrificed by exsanguination and the collected blood was analyzed for E2 content as described above. Xenografts collected at each time point were processed for histologic analysis (n = 8), whereas at day 70 xenografts were immediately snap-frozen in liquid nitrogen (n = 6) and stored at −70°C for subsequent extraction of RNA.

Morphometry

The cellular composition of xenograft tissue was examined by morphometry at each of the harvest time points (n = 8). Sections stained with H&E were viewed at ×250 magnification through a 10 × 10 grid. Tissue was scored at the intersections of the grid as epithelium, fat, or stroma (including fibroblasts and endothelium). A minimum of three fields of view were scored from one E2-treated and one untreated section at each time point from a total of eight implanted human samples.
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of E2 at both days 21 and 42 but returned to baseline at day 70 (Fig. 4A). There were no significant differences between control groups at any of the time points. Proliferation of epithelial cells, as assessed with the antibody marker Ki67, was significantly higher following 7 and 28 days of E2 administration (days 21 and 42; Fig. 4B). Following E2 withdrawal by removal of the pellet, the proliferation of treated samples decreased to baseline levels. There were no significant differences between the control untreated tissues at any of the time points. These data suggest that serum levels of E2 that mimic those seen during human pregnancy induce progesterone receptor expression and elicit a proliferative response in the epithelial cells leading to increases in epithelial content of the tissue. Following E2 withdrawal, proliferation and E2-induced progesterone receptor expression decrease to baseline levels. The increase in epithelial content seen during the induced pregnancy-like changes is also lost at this time.

Expression analysis reveals overlap between the human and rodent genes regulated by pregnancy

Quantitative reverse transcription-PCR was used to analyze the expression of genes previously identified as pregnancy-induced in rodents (11, 12, 19–21). Expression was compared in xenografted human tissue from animals treated with E2 versus tissue from untreated animals (Fig. 5). The numbers of samples retrieved at 72 days posttransplantation ranged from three to six xenografts for each of the six patients (Supplementary Table S3). Gene expression was measured in RNA obtained from individual xenografts and no tissue was pooled for analysis. Data were pooled from the two higher serum E2 doses and showed that 10 of 19 selected genes were found to be differentially regulated by real-time PCR. Up-regulated genes included TGFβ1 (3.34-fold), decorin (DCN; 2.56-fold), cell division cycle 42 (CDC42; 2.06-fold), amphiregulin (AREG; 1.95-fold), keratin 14 (KRT14; 1.94-fold), and lactotransferrin (LTF; 1.90-fold), suggesting increases in growth factors, differentiation markers, and ECM components. Down-regulated genes included connective tissue growth factor (CTGF; 0.52-fold), signal transducer and activator of transcription 1 (STAT1; 0.61-fold), and vesicle-associated membrane protein 1 (VAMP1; 0.69-fold). On exclusion of the sample from the oldest nulliparous patient (age 34), insulin-like growth factor I (IGF1) was also differentially down-regulated (0.53-fold). Thus, these data indicated decreases in cytokine/growth factor signaling following exposure of nulliparous breast tissue to human pregnancy serum E2 levels.

Epithelial STAT1 protein levels are reduced in breast tissues of parous women

To validate gene expression data derived from our in vivo model of human breast tissue, we performed STAT1 immunohistochemistry on 30 age-matched parous and nonparous specimens (Fig. 6). The median percentage of cells scored positive for STAT1 in the parous group was 23.9% (interquartile range, 16.6–33%), which was significantly lower than the median percentage for STAT1 in the nulliparous group of 41.8% (interquartile range, 21.7–55.4%; P < 0.05, Mann-Whitney U test). This suggests that reduction in epithelial STAT1 protein levels is a good marker of parity-induced changes.

Discussion

The protective effect of an early full-term pregnancy has been shown in both human epidemiologic studies and in vivo experiments in rodents. This protective effect can be mimicked in rodent models by treatment with pregnancy levels of exogenous E2. Previous work from this laboratory successfully used a mouse xenograft model to investigate menstrual cycle levels of E2 on cell proliferation and gene expression (14–17). Here, we have used this xenograft model to simulate parity by implanting nulliparous human breast tissue in immunocompromised nude mice and treating them with high-dose E2. Treatment of mice with human 2nd trimester pregnancy levels of E2 for a period of 4 weeks was followed by 4 weeks of withdrawal to mimic involution. We identified genes
with differential expression following treatment with and withdrawal from pregnancy E₂ levels. Our experimental approach differs from that of Russo and coworkers (23) who compared gene expression in postmenopausal breast tissue from parous and nonparous women. In contrast, we show gene expression differences induced in premenopausal nulliparous breast tissue by intervention with pregnancy levels of E₂. These genes were previously identified in rodents, suggesting that similar changes are taking place in the two species. Furthermore, we showed that protein expression for one of these genes, STAT1, could be measured by immunohistochemistry in tissue sections of normal breast epithelium and that differences between age-matched parous and nonparous women could be detected.

All 10 genes identified as being differentially expressed by real-time PCR in this study have been previously identified as differentially expressed in animal models (11–13). The direction of change in expression, either up or down, was also the same with the exception of AREG. AREG has a major role in the promotion of breast cell proliferation and is known to be induced by estrogen in the human mammary gland (17). It is thought to act as a paracrine regulator of

Fig. 3. Tissue composition of implanted human breast xenografts. Changes in composition of implanted tissues over the time of the experiment. The numbers by the error bars represent significance values for differences between time points. A, stroma. 1, differences between day 14 and day 70 within the control group \( (P < 0.001) \); 2, differences between the control group and the treated group at day 42 \( (P < 0.01) \); 3, differences within the treated group between day 14 and day 70 \( (P < 0.0001) \). B, epithelium. 1, difference between the control and treated groups at day 42 \( (P < 0.01) \). C, adipose. Difference between day 14 and day 70 in both the control \( (1, P < 0.0001) \) and treated \( (2, P < 0.0001) \) groups.

Fig. 4. Expression of progesterone receptor and Ki67 (proliferation) in human breast tissue xenografts removed from nude mice. Xenografts harvested at days 14, 21, 42, and 70 were processed for standard immunohistochemistry (IHC). Scoring is expressed as percentage of total epithelial cells positive for the respective marker. Numbers by the error bars represent significance values for the differences between time points. A, progesterone receptor \( (PR) \). Differences within the treated group: 1, between day 14 and day 21 \( (P < 0.01) \); 2, between day 14 and day 42 \( (P < 0.01) \); 3, between day 21 and day 70 \( (P < 0.001) \); 4, between day 42 and day 70 \( (P < 0.01) \). Differences between the treated and control groups: 5, day 21 \( (P < 0.05) \); 6, day 42 \( (P < 0.01) \). B, the proliferation marker Ki67. Differences within the treated group: 1, between day 14 and day 21 \( (P < 0.01) \); 2, between day 14 and day 42 \( (P < 0.01) \); 3, between day 21 and day 70 \( (P < 0.01) \); 4, between day 42 and day 70 \( (P < 0.01) \). Differences between the treated and control groups: 4, day 21 \( (P < 0.01) \); 5, day 42 \( (P < 0.05) \).
estrogen signaling (24). Expression of areg has been shown to increase during pubertal development and to decline during late pregnancy and lactation in rodents (25). Previous animal models of the protective effect of parity have indicated down-regulation of AREG, and the authors have speculated that the suppression of its growth-promoting properties may be a contributory factor in the protective effect (12, 13). Our finding of up-regulated AREG may indicate that its expression diminishes more slowly in humans than in rodents following decreases in E2 levels. However, we show that the growth of epithelial cells has returned to baseline, nonpregnant levels at this time point, suggesting that the sustained AREG levels are no longer stimulating pregnancy levels of epithelial cell proliferation. One explanation for this may be that expression of growth inhibitory growth factors such TGFβ3 are increased after treatment with E2. TGFβ3 is a local apoptosis inducing or growth inhibiting factor, which is expressed in all stages of mammary development with the exception of lactation (26). In addition to the increase of a growth inhibitory factor, the expression of two growth-stimulatory factors was decreased by pregnancy levels of E2. CTGF is known to be estrogen inducible and is overexpressed in steroid-dependent breast and uterine tumors (27). It is thought to be an important downstream mediator of estrogen and progesterone-regulated cell growth although it may affect other growth regulatory pathways in breast cancer cells (27). The other down-regulated gene was IGF1, which functions along with growth hormone and estrogen in postpubertal breast development and has been shown to be a survival factor in breast (28). Thus, our results suggest that down-regulation of growth factors and up-regulation of growth inhibitory factors by parity may contribute toward its protective effect.

In addition to growth factors, we found that genes involved in ECM, tissue remodeling, and differentiation were modulated by pregnancy levels of E2. For example, we found increased levels of DCN expression after treatment with E2. There is some evidence to suggest that ECM components such as collagen and proteoglycans may be the primary determinants of mammographic density, which is, in turn, related to breast cancer risk (7, 19, 29). Therefore, we might speculate that pregnancy-associated changes in ECM components such as DCN may reduce breast density.

We found increased expression of CDC42, which is a member of the Rho GTPase family of proteins and performs important functions in cell migration (30). Migration is likely to be increased during remodeling of the breast tissue, which takes place following pregnancy. We also found increased expression of two differentiation markers, LTF and KRT14.

Fig. 5. Quantitative real-time PCR. RNA was extracted from xenografts harvested at day 70 from both treated and control animals, amplified, and then reverse transcribed to cDNA. Columns, mean fold changes in gene expression between control and treated samples, calculated as described in Materials and Methods. Columns to the right of the dotted line represent the genes considered to be differentially regulated, which was judged to be when the mean less the SE remained >1-fold. The hatched column was found to be differentially regulated in all but one of the implanted tissues, whereas the genes represented by dark gray columns were differentially regulated in all samples.

Fig. 6. Comparison of staining for STAT1 by immunohistochemistry between age-matched samples of archival human breast tissue. A, percentage of cells positive was significantly higher in nulliparous tissue as compared with parous tissue (Mann-Whitney U test, P = 0.042). Photomicrographs illustrating differing staining patterns observed in nulliparous (B) and parous (C) samples (bar, 50 μm). Cells with brown staining nuclei were scored as positive.
Finally, we identified STAT1, the intracellular signaling component downstream of IFNs, to be highly down-regulated by pregnancy E2 levels in our model. Importantly, we confirmed that a down-regulation in STAT1 protein occurs in the breast epithelial cells of parous compared with nonparous women of the same age (Fig. 6). STAT1 reduction in parous breast tissue suggests a decrease in the proportion of cells that could respond to IFN. Interestingly, IFN-α has recently been shown to induce hematopoietic stem cell activity (31), and one could speculate that epithelial stem cell activity in breast tissue is altered and reflected in STAT1 expression. This finding of reduced STAT1 expression in parous compared with nonparous women suggests that our xenograft model is a good simulation of hormonal effects during human pregnancy and reveals STAT1 as a potential biomarker of pregnancy-induced breast cancer protection.

It has been suggested that possible mechanisms of pregnancy-induced protection include changes to the hormonal profile of parous women, changes in epithelial responses to hormones, a more differentiated and thus less susceptible mammary gland, or changes within specific epithelial cell subpopulations (32). Our results do not test whether there are sustained systemic hormonal changes, although serum E2 levels were found to return to baseline after treatment was withdrawn for 4 weeks. At this time, we also determined whether the number of cells expressing estrogen receptor or progesterone receptor had changed and found no evidence for this, although changes in the quantity of receptors per cell was not measured and could influence response to hormones. We did see an increase in KRT14, perhaps indicating an increase in the proportion of basal cells, but we have not used any markers to identify specific changes in stem, basal, myoepithelial, or luminal cell number in our study.

It is also well founded epidemiologically that there is an increase in risk of breast cancer shortly after pregnancy (up to 10 years) and that it is after this period that the protective effect is observed (33). This suggests that the genes that change in the short term, which our model measures, may be associated with an increased, rather than a decreased, risk of breast cancer. This paradoxical finding may confound our search for markers of decreased risk. However, the identification of STAT1 as down-regulated both in our model shortly after withdrawal from pregnancy E2 levels and in parous women some years after pregnancy indicates that valuable markers of pregnancy-induced protection can be identified.

In conclusion, we have identified changes in gene and protein expression in nulliparous, normal human breast tissue treated with pregnancy levels of E2 and correlated these with the effects of parity in premenopausal women. Our results show that many of the pregnancy-induced breast cancer–protective changes observed in rodent models also occur in human breast tissue following estrogen intervention and that STAT1 expression is a potential biomarker of parity-induced breast cancer protection in the human breast.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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