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The Proliferation of Normal Human Breast Tissue Implanted into Athymic Nude Mice Is Stimulated by Estrogen but Not Progesterone

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ABSTRACT

In order to resolve the question of which ovarian steroid stimulates normal human mammary epithelial cell proliferation, we have implanted pieces of normal human breast tissue subcutaneously into athymic nude mice. These mice were then treated with slow-release pellets containing estradiol (E_2) or progesterone (P) such that serum levels of E_2 and P were increased to those seen in normal women. The proliferative activity of the tissue implants was assessed by uptake of tritiated thymidine and steroid receptor expression was measured immunocytochemically.

Insertion of a 2 mg E_2 pellet 14 days after tissue implantation increased the thymidine labeling index (TLI) from a median of 0.4% (n = 34) to a median of 2.1% after 7 days (n = 43; $P < 0.001$ by Mann Whitney U test). In contrast, treatment with a P pellet (4 mg) had no effect upon the TLI whereas P (4 mg) in combination with E_2 (2 mg) had no effect over and above that of E_2 alone. There was a significant correlation between the increase in TLI and either the E_2 content of the pellets ($P < 0.001$ by linear regression) or the serum E_2 levels achieved ($P < 0.001$). Expression of the P receptor was increased 15- to 20-fold by E_2 treatment.

We conclude that E_2 is sufficient to stimulate human breast epithelial cell proliferation at physiologically relevant concentrations and that P does not affect proliferation either alone or after E_2 priming. (*Endocrinology* **136:** 164–171, 1995)

THE FACTORS controlling proliferation of normal human mammary epithelial cells *in vivo* are not well understood. We, and others, have shown that the rate of proliferation of epithelial cells in the normal human mammary gland varies throughout the menstrual cycle (1–7). These cyclical changes do not occur in the absence of an intact anterior pituitary gland or the ovaries suggesting the involvement of both peptide and steroid hormones. The most obvious candidate steroid hormones are estradiol (E_2) and progesterone (P) since they are produced in a cyclical fashion by the ovaries (8). In the endometrium, a classical estrogen target tissue, epithelial cell proliferation is maximal in the follicular phase of the menstrual cycle and declines rapidly when serum P levels rise in the luteal phase of the cycle (9). In direct contrast, breast epithelial cell proliferation, as measured by thymidine labeling, is at its lowest in the follicular phase and at its highest in the luteal phase at a time when the endometrium is secretory in appearance (1–7, 9). These findings have led several groups to suggest that P either alone or after estrogen priming is the major steroid mitogen for normal human mammary epithelium (10–12).

The steroid responsiveness of normal human mammary epithelial cells has been studied in primary culture. One group demonstrated increased growth of the cells in response to E_2 that could be inhibited by tamoxifen treatment (13). In a separate study this group also showed an inhibitory effect of progestins on the growth of human breast epithelial cells in primary culture (14). However, other studies have not found any effect of E_2 upon human breast epithelial cell proliferation *in vitro* (15). These findings are surprising in the light of evidence showing that human breast (and breast tumor) development does not occur in the absence of estrogen (16). The *in vitro* findings are also at variance with other observations showing a significant positive relationship between breast proliferation and the dose of estrogen in women taking the oral contraceptive pill (11). Finally, extensive studies of malignant breast epithelial cells both *in vitro* and *in vivo* show that E_2 is a major mitogenic stimulus (17, 18) whereas P is often found to have only minor additive or even inhibitory effects upon proliferation (reviewed in 19).

We have attempted to resolve the question of which ovarian steroid stimulates normal human mammary epithelial cell proliferation by implanting pieces of human breast tissue sc into athymic nude mice. We assessed the effects of E_2 and P on proliferation, both individually and in combination, by measuring the thymidine labeling index (TLI). This model has the advantage of preserving the normal tissue architecture and the parenchymal interactions with the surrounding stroma and is similar to that developed by McManus and Welsch (20, 21). However, it is uncertain whether the tissue used by this group was completely histologically normal as

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it was obtained from women with diffuse benign lesions. In addition, the serum steroid levels achieved by the implants used by McManus and Welsch (20, 21) were not estimated. In our studies, only histologically normal breast tissue was used for implantation and the mice were treated with slow release silastic pellets that were shown to deliver levels of E_2 and P comparable to those seen in the serum of women during the menstrual cycle and pregnancy. Using this model we have shown that proliferation of the implanted human breast tissue was stimulated in a dose-dependent manner by E_2 but not P. Moreover, the effects of P combined with E_2 (in a manner that approximated the human menstrual cycle) were no different from those of estrogen alone.

Materials and Methods

Patients

Eighty nine women (median age 27.1 yr) with a lesion in the breast requiring diagnostic biopsy were studied. In order to enter the study, a history of regular menstrual cycles was required. Women taking the oral contraceptive were not excluded (n = 17). The date of the menstrual cycle on which the biopsy was performed was assessed by 1) the patient noting the first day of their last menstrual period, 2) noting their normal cycle length, and 3) by asking the women to return a prepaid card indicating the first day of their next menstrual period. Serum levels of E_2 and P were determined in blood samples taken on the day of surgery. The study protocol was approved by the South Manchester Ethics Committee and all patients gave informed consent for the study.

At the time of operation, a biopsy of macroscopically normal tissue was taken from a site approximately 1 cm away from the lesion. The lesions consisted of fibroadenomas (n = 67), ductal carcinoma *in situ* (n = 2), fibrocystic disease (n = 6), adenosis (n = 1), fibroadenomatoid hyperplasia (n = 2), and sclerosing adenosis (n = 1) whereas the remaining 10 biopsies contained only normal tissue.

The women's menstrual cycles varied in length between 23 and 33 days and the serum E_2 and P levels determined on the day of operation were consistent with the stage of the menstrual cycle at that time. The day of cycle was adjusted to a standard 28-day pattern in those women with longer or shorter cycles on the assumption that variation in the length of the menstrual cycle was due to changes in the length of the follicular phase whereas the luteal phase length remained a constant 14 days (22).

Animals

All the animals used were 9- to 10-week-old intact female athymic nude (Balb/c nu/nu) mice, bred in the colony maintained at this institute. The experimental mice were housed under conventional conditions with a 12-h cycle of light and dark (lights off 1900–0700 h) in filter top cages supplied *ad libitum* with irradiated feed together with autoclaved water and bedding. All surgical procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and halothane inhalation anesthesia (2–4% halothane in oxygen; Halovet Vapouriser, International Market Supplies, Cougleton, UK) was used for all procedures.

Materials

Except where specified, all chemicals used in this study were of analytical grade or higher and were obtained from either Sigma (Poole, UK) or BDH (Lutterworth, UK). Tritiated thymidine (186 GBq/mmol; 5 Ci/mmol) was supplied by Dupont New England Nuclear, UK.

Treatment of tissue samples

Each tissue sample was stripped of excess fat and divided into several portions of approximately equal size. Each was sliced under sterile conditions into eight pieces of approximately 2 × 1 × 1 mm which was then placed in Dulbecco's Modified Eagle's Medium (GIBCO, Paisley, UK) until implantation into the nude mice. Another portion of tissue was cut into 1-mm wide strips and used for measurement of proliferative activity by uptake of tritiated thymidine (see below). The remaining pieces were fixed in Carnoy's fixative, paraffin-embedded, sectioned, and then stained with hematoxylin and eosin for histological examination. Only histologically normal breast tissue was used and if abnormalities were found, the experiment in the nude mice was terminated. Where there was sufficient material, some tissue was frozen in liquid nitrogen for subsequent assessment of estrogen and progesterone receptor (ER and PR) content.

Implantation of breast tissue into nude mice

Each patient's sample was divided between four mice. Two small incisions were made across the midline dorsal skin through which eight tissue pieces were symmetrically placed. To retrieve these xenografts at the end of the experimental period, the mice were reanesthetized and each graft was excised using sharp dissection. The grafts were then processed for thymidine labeling or histology as described below. Blood was taken from each of the mice at the conclusion of the experiment from which serum was prepared and stored at −70 C until assay.

Steroid administration

Similar methods were used for the administration of exogenous E_2 and P to the athymic nude mice. Silastic pellets were prepared by mixing dry steroid powder with 382 Medical Grade Elastomer (BDH). Sufficient steroid was added to the silastic elastomer to yield a final weight of 0, 0.5, 1, 2, or 6 mg E_2 or 4 mg P/pellet, respectively. The silastic-steroid mixture was then thoroughly mixed before the catalyst was added and the mixture spread into a glass template to make a 1-mm thick sheet. The individual pellets were formed by cutting the sheet into 4 × 4-mm squares which were stored dry until use, at which time they were inserted sc into the flanks of the mice well away from the site of tissue implantation.

Thymidine labeling

Pieces of normal breast tissue (strips ~1 mm wide) obtained from either the patients or the mice were incubated with Hank's Balanced Salt Solution (GIBCO) containing 37 kBq/ml (1 μCi/ml) tritiated thymidine (^{3}HTdR, SA = 186 GBq/ml; 5 Ci/mmol) for 1 h at 37 C. At the end of this period, the pieces of tissue were fixed in Carnoy's fixative and stored in 70% ethanol until processing for autoradiography when they were embedded in paraffin wax, sectioned, and mounted onto gelatin-coated glass slides. The sections were dewaxed in xylene and then rehydrated through graded alcohols before being dipped in photographic emulsion (K5, Ilford, Mobberley, UK) at 50 C, dried, and stored at 4 C in light-proof conditions for 7 days. After exposure, the slides were developed in D19 developer (Kodak Ltd., Hemel Hempstead, UK) diluted 2-fold with distilled water at 20 C. Development was halted by transfer to a stop bath of 1 M acetic acid and the slides were then fixed for 6 min in Amfix (May and Baker, Eccles, UK) diluted 5-fold with distilled water. After washing for at least 1 h in running tap water, the sections were counterstained with hematoxylin and eosin. In order to avoid grain fading, the developed autoradiographs were stored dry until the day of scoring at which time they were mounted in XAM neutral mounting medium.

Areas for counting were selected out of focus at low power and then complete high power fields were scored. At least 1000 epithelial cells per specimen were counted and the number of labeled cells were expressed as a percentage of the total number of cells counted. A cell was considered labeled if 5 or more silver grains were seen overlying the nucleus.

Histological studies

The histological features of all the specimens were assessed on representative sections stained with hematoxylin and eosin. Specifically, preservation of the normal lobular architecture was assessed together

with an estimation of the number of mitotic cells and the degree of vascularity.

Assessment of ER and PR expression

The ER and PR assay kits (ER-ICA and PR-ICA) supplied by Abbott Laboratories (Maidenhead, UK) were used to stain frozen sections of samples removed at surgery and those obtained after implantation into the mice. Sections were deemed scoreable if an adjacent section stained with hematoxylin and eosin showed normal ducto-lobular architecture and there were clearly identifiable epithelial cells. Fields for scoring were selected out of focus at low power and then cells were counted at high power using a graticule. The intensity of staining was not assessed and cells were judged to be either positive or negative. At least 1000 epithelial cells were counted for each sample and the overall percentage of positive cells was calculated for each specimen.

RIA of serum estrogen and P content

Serum E_2 concentrations were measured using an in-house specific RIA based on a rabbit polyclonal antiserum raised against estradiol-6-(*O*-carboxymethyl) oxime-BSA after extraction of the steroid from the serum using ethyl acetate. The intraassay coefficient of variation (COV) was less than 5% for this assay whereas the interassay COV was 13%.

All serum P measurements were performed by the University Department of Pathology at the University Hospital of South Manchester using the Amerlex-M P RIA kit supplied by Amersham International plc (Amersham, UK). The serum samples obtained from the nude mice were first extracted with ethyl acetate and the extract was reconstituted in charcoal-stripped human serum. Using this extraction technique, the intraassay COV was less than 5% and the interassay COV was 14%.

Statistical methods

In most cases the results are presented as medians together with their respective interquartile (IQ) ranges and statistical comparisons have been made using nonparametric tests (Wilcoxon's Matched-Pair Signed Rank test, Mann Whitney *U* test). Where the data from several experiments have been combined, the results have been standardised by subtracting the TLI obtained on day 14 of the experiment (*i.e.* before initiation of hormone treatment) from the value obtained at the end of the experimental procedure. Standard linear regression was carried out as a test for trend when assessing the relationship between increasing doses of hormone and the change in TLI.

Results

Histology

The histology of all the breast tissue samples used for implantation was reviewed by one pathologist. The presence of microcysts was accepted as part of the range of normal features but larger cysts or disruption of the normal lobular architecture were not and the results from experiments using these particular samples were discarded. The majority of the grafts (58%) showed normal lobular architecture upon recovery from the mice. Twenty eight percent were entirely absent from the site of implantation, whereas 15% showed disrupted morphology consistent with necrosis or fibrosis with no epithelial elements present.

Normal lobular architecture was preserved for up to 56 days after implantation of the xenografts in the absence of steroid supplementation. There was no association between the type of treatment the mice received and the ability to recover xenografts containing normal lobules. Between 50% and 70% of the xenografts showed dilatation of intralobular vascular spaces which contained erythrocytes of murine origin. However, the proportion of grafts which developed a mouse vascular supply was not related to the treatment that was administered nor was there any correlation between the degree of vascularity and the level of proliferation in the implants.

Serum steroid hormone levels

Before hormone treatment the median serum E_2 concentration in the athymic nude mice was 94 pmol/liter with an IQ range of 83–99 pmol/liter. The median serum P concentration was 4 nmol/liter (IQ range = 0–6 nmol/liter). Figure 1 shows the time course of release of steroid from pellets containing either 2 mg E_2 or 4 mg P. Serum levels of E_2 reached between 1200 and 1550 pmol/liter 2 days after insertion and remained at this level for the rest of the 30-day study period (Fig. 1a). Serum P concentrations reached their maximum 3 days after insertion of the pellet and remained at between 35 and 57 nmol/liter for at least 14 days (Fig. 1b). Serum E_2 levels were also measured in the mice 2 weeks after the insertion of pellets containing 0.5, 1, and 6 mg E_2. These pellets produced median serum E_2 levels of 400, 540, and 4400 pmol/liter, respectively (see Fig. 2). In addition, there was a highly significant positive correlation between the pellet size used and the serum E_2 concentration that was achieved ($r = 0.978$; $P < 0.0001$ by linear regression).

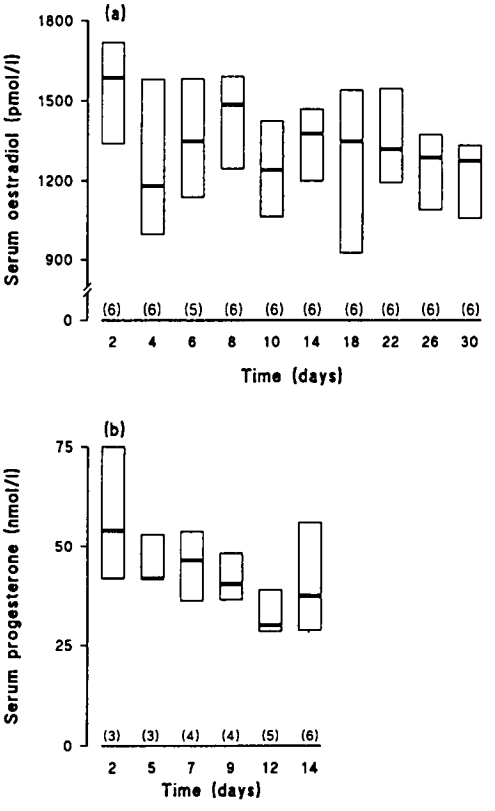

FIG. 1. The steroid release characteristics of silastic pellets containing 2 mg E_2 (a) or (b) 4 mg P. The *columns* indicate the interquartile range of each measurement whereas the *bars* represent the medians. The *numbers in parentheses above the abscissa* are the number of observations in each group.

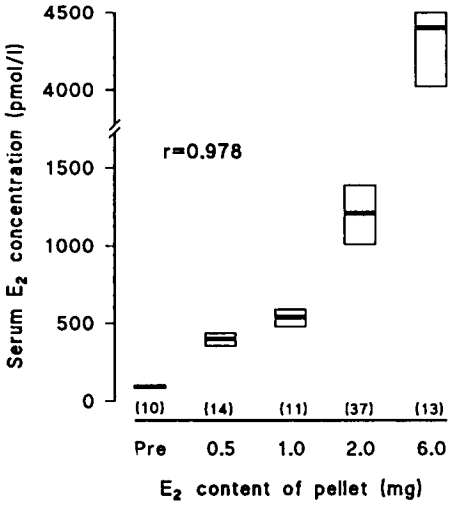

FIG. 2. The serum E_2 concentrations achieved 2 weeks after insertion of silastic pellets containing differing amounts of the hormone. There was a significant correlation between the serum E_2 concentration achieved and the size of the E_2 pellet ($r = 0.978$; $P < 0.001$ by linear regression). The *columns* indicate the interquartile range of each measurement whereas the *bars* represent the medians. The *numbers in parentheses above the abscissa* are the number of observations in each group. Pre = serum E_2 level before insertion of the pellets.

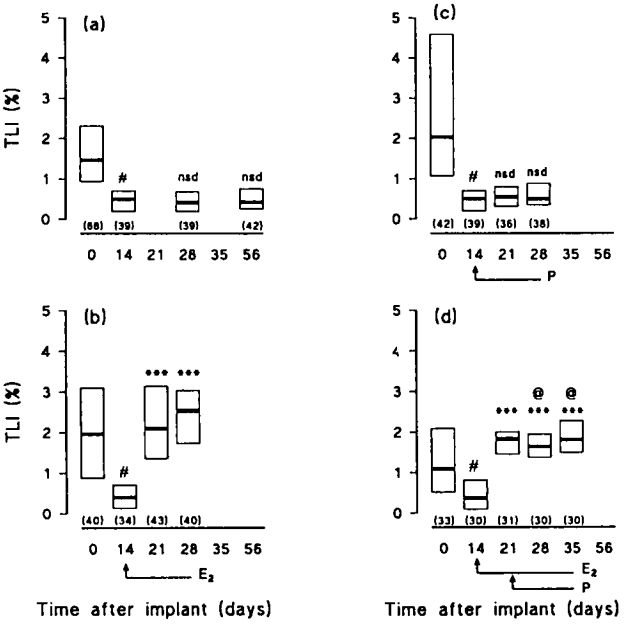

FIG. 3. Proliferative activity of human breast tissue implanted into nude mice that were (a) left untreated; (b) that received a 2-mg E_2 pellet; (c) that received a 4-mg P pellet or (d) that received both an E_2 and a P pellet. The *columns* indicate the interquartile range of each measurement whereas the *bars* represent the medians. The *numbers in parentheses above the abscissa* are the number of observations in each group whereas the *arrows* indicate the duration of steroid treatment. #, Significantly different from the day 0 value ($P < 0.001$ by Mann Whitney *U* test); nsd, not significantly different from the day 14 value; @, not significantly different from the day-21 value.

Proliferation of breast tissue before implantation

The TLI was determined for all the tissue samples used in this study immediately after they were removed from the patient and the values obtained were related to the stage of the menstrual cycle. Although there was wide variation between individuals, the TLI increased from a median of 1.14% (IQ range = 0.79–2.1%; n = 43) in the follicular phase of the cycle to 1.85% (IQ range = 1.24–2.76%; n = 56) in the luteal phase ($P = 0.011$ by Mann Whitney *U* test). The TLI of these samples of normal tissue also showed the expected inverse correlation with the age of the patients ($P < 0.028$ by linear regression).

Effects of steroid treatment on proliferation

Figure 3a shows serial TLI measurements in normal breast tissue after implantation into untreated control mice. The median TLI was reduced from 1.5% to 0.49% after 14 days. After 28 days, the median TLI was 0.41% and it remained at a similar level for the rest of the experiment (*i.e.* for up to 56 days). In subsequent experiments the mice were left untreated for 14 days after tissue implantation in order to allow proliferation to fall to basal levels. At this time, some samples of tissue were removed from the mice and the hormone-silastic pellets were inserted. Further tissue samples were removed at the indicated times after the start of treatment. The effects of treatment with a pellet containing 2 mg E_2 are shown in Fig. 3b. There was a significant rise to a TLI of between 2% and 3% after 7 days of treatment, *i.e.* an approximate 6- to 8-fold increase over control levels seen before administration of the pellet ($P < 0.0001$ by Mann Whitney *U* test). The TLI remained at this level for at least 14 days after the start of treatment. Figure 3c shows that insertion of a 4 mg P pellet alone did not alter the TLI from that seen before treatment even after 14 days of treatment. Similar results were obtained when 12 mg P were administered (data not shown). Finally, E_2 given for 3 weeks supplemented with P for the last 2 weeks, in a manner that approximated the pattern of the human menstrual cycle, had no significant effect over the effects of E_2 alone (Fig. 3d).

The response to E_2 was examined in more detail by determining the time course of the increase in TLI after treatment and also by examining the relationship between the effects on TLI and either the pellet size or the serum E_2 concentrations that were achieved. Figure 4 shows the results of daily measurement of the TLI in the normal tissue xenografts after insertion of a silastic pellet containing 2 mg E_2. The increase in TLI caused by this treatment became significant 3 days after insertion of the pellet and was maximal at 6–7 days.

Figure 5a shows that the magnitude of the change in TLI caused by 14 day's treatment with E_2 was highly significantly correlated to the amount of E_2 in the silastic pellet. Furthermore, a significant, relationship could be demonstrated between the change in TLI and the log of the serum E_2 levels achieved after insertion of the steroid pellets (Fig. 5b).

Effects of steroid treatment on expression of ER and PR

The changes in breast epithelial cell steroid receptor expression are shown in Fig. 6. None of the treatments had any significant effect upon the proportion of cells that expressed ER (Fig. 6a). However, a 15- to 20-fold increase in the number

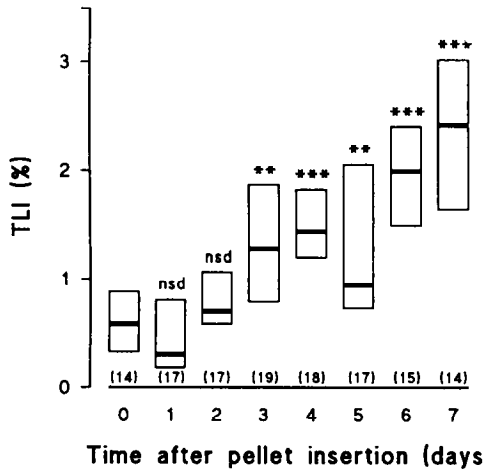

FIG. 4. The time course of the increase in proliferative activity of implanted normal human breast tissue in response to treatment with a 2-mg pellet of E_2. The *columns* indicate the interquartile range of each measurement whereas the *bars* represent the medians. The *numbers in parentheses above the abscissa* are the number of observations in each group. nsd, Not significantly different from the day 0 value by Mann Whitney *U* test; **, significantly different from the day 0 value ($P < 0.01$); ***, significantly different from the day 0 value ($P < 0.001$).

of cells expressing PR was seen 7 days after insertion of a 2 mg E_2 pellet ($P < 0.001$ by Mann Whitney *U* test). The level of PR expression was significantly reduced after 14 days of E_2 treatment compared to that seen at 7 days although it was still much higher than that seen in the absence of treatment. There was no effect of P alone and combined treatment with E_2 and P had no further effect over and above that seen after 14 day's treatment with E_2 alone.

Discussion

In this study we have attempted to resolve the question of whether E_2 or P is the major mitogenic ovarian steroid for normal human mammary epithelium. In order to do this, we have adopted the model described by McManus and Welsch (20, 21) which allows hormonal manipulation of normal human breast tissue after it has been implanted into immune-deficient athymic nude mice. We chose to implant pieces of tissue rather than disaggregated organoids for practical reasons in that the time required to process the tissue is rather less than that required to produce enzymatically dispersed organoids as used by other groups (23, 24). This method has the advantage of preserving the normal tissue architecture and, presumably, normal epithelial-mesenchymal interactions. We have, however, refined the model of McManus and Welsch (20, 21) in that we have manipulated serum hormone levels in the mice so that they approximate those seen in normal premenopausal women. We have also ensured that the breast tissue removed as far away as possible from the site of a discrete benign lesion was used and we discarded the results from pieces of tissue that were shown to have abnormal features upon subsequent histological examination. Despite these precautions, we were unable to attain the 100% "take rate" achieved by McManus and Welsch. Our lower take rate may be related to the increased width of the

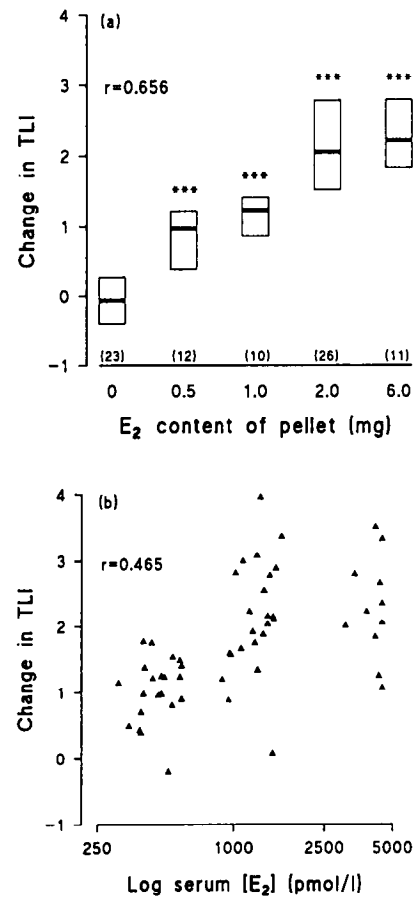

FIG. 5. The relationship between the change in TLI of human breast tissue implanted into athymic nude mice and (a) the size of the E_2 pellets with which the mice were treated or (b) the serum E_2 concentration measured at the time of removal of the tissue from the mice. The *columns* indicate the interquartile range of each measurement whereas the *bars* represent the medians. The *numbers in parentheses above the abscissa* are the number of observations in each group. ***, Significantly different from the 0-mg value by Mann Whitney *U* test ($P < 0.001$). The change in the TLI and the size of the E_2 pellet were significantly correlated by linear regression ($n = 82$; $P < 0.001$). The change in TLI was also significantly correlated to the serum E_2 level ($n = 55$; $P < 0.001$).

tissue sections we used compared to the studies of McManus and Welsch (20, 21) and, second, the nature of the benign lesions that they accepted which may have affected persistence of the grafts. We found no evidence to indicate that either the take rate or the histological integrity of the xenografts was related to the type or dose of steroid supplementation given and this is in accordance with previous studies. Likewise, some of the xenografts demonstrated evidence of vascularization from the mouse which was not related to the steroid treatment or the response to hormonal stimuli.

Measurement of serum E_2 and P levels in the untreated nude mice used in our study confirmed that they were equivalent to those of normal postmenopausal women (25). Serum hormone concentrations achieved with the silastic pellets containing 2 mg E_2 were comparable to those seen at the human midluteal peak (8) and remained at this level for at least 30 days. The pellets containing 0.5 and 1 mg E_2 yielded

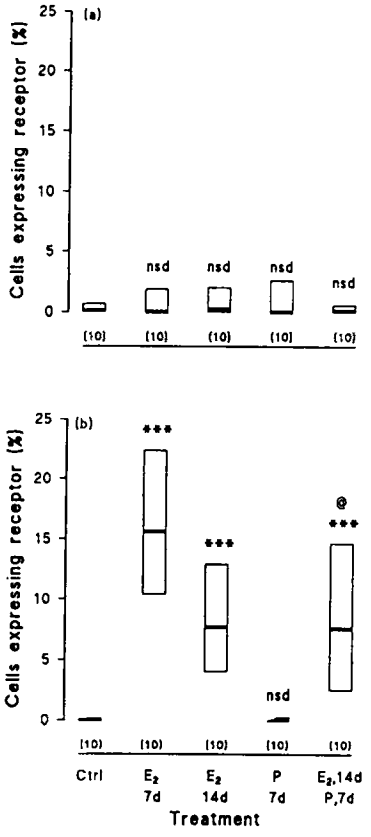

FIG. 6. The effects of E_2 and P treatment on the expression of ER (a) and PR (b) in human breast tissue implanted into athymic nude mice. The *columns* indicate the interquartile range of each measurement whereas the *bars* represent the medians. The *numbers in parentheses above the abscissa* are the number of observations in each group. Ctrl, No treatment; E_2,7d = 7 days' treatment with a 2-mg E_2 pellet; E_2,14d = 14 days' treatment with a 2-mg E_2 pellet; P,7d = 7 days' treatment with a 4-mg P pellet; $E_2$14d,P7d = treatment with a 2-mg E_2 pellet for 14 days combined with a 4-mg P pellet for the final 7 days. nsd, Not significantly different from the Ctrl value; ***, significantly different from the Ctrl value ($P < 0.001$ by Mann Whitney U test); @, not significantly different from the E_2,14d value.

levels that were similar to those seen in the midfollicular phase in normal women whereas the 6-mg pellet increased the serum E_2 concentration to levels far in excess of those found throughout the normal human menstrual cycle and approaching those seen in pregnancy (8). The mouse serum P levels achieved with a pellet containing 4 mg steroid were approximately equivalent to those at the human midluteal phase peak. These levels could be maintained for at least 14 days which was the duration of the longest experiment involving P treatment. Simultaneous insertion of three pellets containing 4 mg P produced supraphysiological serum levels of P which far exceeded those found throughout the human menstrual cycle. The use of slow release estrogen silastic pellets has been criticized on the grounds that they do not produce a full lactogenic response (23). The aim of the present study was not to produce a lactogenic response but to determine the factors controlling breast epithelial cell proliferation during the menstrual cycle. The silastic-hormone pellets provide a means of tightly controlling both the amount and the duration of steroid administration to the mice. This differs from the model of Gusterson *et al.* (23) in which the mice were made pregnant in order to stimulate the normal breast epithelial cells and that of McManus and Welsch (20, 21) in which slow release cholesterol-E_2 pellets were supplemented by addition of estrone to the drinking water and grafting of pituitary tumors secreting unknown quantities of PRL and GH.

As expected from previous studies (1–7), the proliferative activity of the normal breast tissue biopsy samples measured at removal from the patients showed considerable variation. In keeping with previous findings, the median TLI was higher in tissue removed in the luteal phase of the menstrual cycle compared to that taken during the follicular phase (1–7). Furthermore, the TLI of the operative specimens was inversely correlated with the age of the patients and this is also in agreement with the results of previous investigations (1, 3). Measurement of the proliferative activity of the breast tissue xenografted into untreated mice showed that, irrespective of the initial level of proliferation, the TLI fell to very low levels by day 14 after implantation. This level of proliferation did not fluctuate significantly during the rest of the experimental period in the absence of hormonal supplementation (56 days) implying that the mitogenic factors responsible for human breast epithelial cell proliferation are not present in sufficient quantity in the intact female athymic nude mouse. However it is possible that the low level of proliferation in the implant at this time was due to the low levels of endogenous steroid hormones in the mice. Histological examination of the tissue removed after 56 days in the mice showed that it remained intact with no features suggestive of degeneration and we were able to show that breast tissue was still able to mount a proliferative response to 7 day's treatment with a 2-mg E_2 pellet after 49 days without treatment (data not shown).

E_2 very clearly increased the proliferative activity of the human breast tissue xenografts when measured 7 and 14 days after the start of treatment. All sizes of E_2 pellet (0.5, 1, 2, and 6) significantly increased the TLI compared to that measured before treatment was started. However, since the 2-mg pellet produced serum E_2 levels in the mice that were similar to those seen in the women at the midcycle peak of the menstrual cycle, this pellet size was used to investigate the estrogenic effects in further detail. Nonspecific effects of E_2 on thymidine uptake were first excluded by carrying out vincristine arrest experiments which showed significant accumulation of mitoses during E_2 treatment compared with untreated controls (data not shown). The increase in TLI caused by the 2-mg E_2 pellet appeared to be maximal 7 days after the start of treatment with no further enhancement between 7 and 14 days. The time course of this increase was examined in more detail by measuring the TLI each day after insertion of the E_2 pellet. The results of this experiment showed that the TLI began to rise significantly 3 days after the start of treatment. The time taken to reach maximal proliferation (6–7 days) approximates the time between the midcycle peak of E_2 secretion and the subsequent midluteal phase increase in TLI seen in the normal human breast *in vivo* (1–7).

A dose response effect could be demonstrated between the

E_2 pellet size and the level of thymidine labeling achieved. However, proliferation appeared to reach a plateau as the 6-mg E_2 pellet did not increase proliferation over and above that seen with the 2-mg pellet. These findings are in agreement with those of McManus and Welsch (21) who showed that the LI was increased maximally by a cholesterol pellet containing 5 mg E_2 but was then reduced after treatment with a 10-mg E_2 pellet. We have also related the proliferative activity of the normal breast tissue xenografts to the serum E_2 levels that were achieved by treatment. The magnitude of the change in TLI was significantly related to the serum E_2 concentrations in the mice. Again, there was an apparent plateau in the dose response curve above serum E_2 levels of approximately 1500 pmol/liter (*i.e.* that produced by a 2-mg E_2 pellet). Significant correlations between the breast epithelial TLI and serum E_2 were also demonstrated within each of the treatment groups (data not shown). It is encouraging to note that our conclusions regarding the stimulation of breast epithelial cell proliferation by physiological levels of E_2 are supported by the recent report of Silberstein *et al.* (26). This group used intramammary implants of pure antiestrogens to show that endogenous E_2 is essential for mouse mammary ductal growth and morphogenesis *in vivo*.

In contrast with the stimulatory effects of E_2 on the human breast tissue xenografts, P appeared to be entirely without effect at either physiological or supraphysiological concentrations, alone or in combination with E_2. McManus and Welsch (21) were also unable to demonstrate an effect of P in a very similar nude mouse model. The only difference between the two models was that the mice in the present study were "primed" with E_2 for 7 days before P was administered, whereas McManus and Welsch (21) administered E_2 and P simultaneously. One explanation for the lack of effect of P is that the epithelial cells did not express sufficient PR to elicit a response. Against this, however, is the finding that 7 days of treatment with E_2 caused a 15- to 20-fold increase in the number of cells expressing the PR. Treatment with P at this stage was ineffective suggesting that the hormone is not mitogenic for the human breast epithelium. We also did not obtain evidence for an inhibitory effect of P on the normal mammary epithelium. This is in direct contrast to the human endometrium where proliferation is almost completely inhibited in response to increasing levels of P in the second half of the menstrual cycle (6, 9). It is possible that P could not simulate the TLI because the effect of E_2 was maximal. We cannot exclude this possibility but P alone in the presence of small amounts of endogenous E_2 did not stimulate proliferation.

We, and others, have shown that the percentage of cells which express ER in the normal human breast varies throughout the menstrual cycle (2, 27, 28). Lobules sampled during the follicular phase of the cycle contain detectable ER in 0–15% of cells which falls to 0–5% in the luteal phase (2, 27, 28). We suggested that this fall in ER content may be due to down-regulation of receptor expression by either E_2 or P. In the nude mouse model, however, the number of cells expressing ER after the 14-day control period was very small and subsequent steroid treatment had no additional effect. This result was surprising, but it is possible that the ER-ICA kit was not sufficiently sensitive to detect the low levels of ER expected to be present. This is supported by the fact that tissue was still able to mount a response to E_2 stimulation both in terms of PR up-regulation and increased TLI. The proportion of lobular epithelial cells which expressed the PR was also very small after the 14-day control period. In this case, however, E_2 treatment caused a highly significant rise in the number of cells expressing PR. This is similar to the situation in steroid receptor positive breast cancer cell lines where expression of the PR has been shown to be positively regulated by E_2 (29). P neither stimulated nor inhibited expression of its own receptor as the proportion of PR+ve cells was not altered by administration of P alone or together with E_2. The effects of E_2 treatment on the number of cells expressing PR in the xenografts were surprising. We had previously shown that PR expression in normal lobules removed from women remains high throughout the menstrual cycle and suggested that this was because PR synthesis was constitutive (2). This hypothesis now appears incorrect since in the nude mouse model of normal human breast tissue, lobular PR expression is up-regulated by E_2, but not down-regulated by P.

In summary, we have adopted the model developed by McManus and Welsch (20, 21) in order to study the hormone responsiveness of normal human breast tissue implanted into athymic nude mice. We have, however, refined this model by defining the serum hormone concentrations achieved by the steroid treatment; by ensuring that only histologically normal tissue was used and by validating the TLI measurements with vincristine arrest experiments. E_2 appears to be sufficient to stimulate human breast epithelial cell proliferation at physiologically relevant concentrations and that P does not affect proliferation either alone or after E_2 priming. We also demonstrated a dose response relationship between proliferation and serum E_2 concentrations and, in this model, E_2 also stimulates PR expression. Finally, we suggest that the time course of response to estrogenic stimulation in this nude mouse model may provide an explanation for the pattern of proliferative activity seen in the breast during the menstrual cycle *in vivo*. Although we have mimicked the proliferation seen in human normal breast in the mouse model, we cannot exclude the possibility that the endocrine control of proliferation in women differs from that which we have demonstrated in the mice.

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