Breast Cancer Research and Treatment **79:** 233–239, 2003.

Report

Effects of long-term HRT and tamoxifen on the expression of progesterone receptors A and B in breast tissue from surgically postmenopausal cynomolgus macaques

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Key words: AR, cynomolgus macaques, hormone replacement therapy, PRA, PRB, tamoxifen

Summary

Estrogen is a well-known mitogen in breast epithelium but the role of progesterone is complex and incompletely understood. In contrast to what is seen in the endometrium, combined estrogen/progestogen treatment for postmenopausal replacement (HRT) may carry a risk for breast cancer beyond that of estrogen alone. The ratio of the two progesterone receptor (PR) isoforms, PRA/PRB may define the response to progesterone in reproductive tissues. In a primate model for long-term HRT, surgically, postmenopausal cynomolgus macaques were treated for 35 months with conjugated equine estrogens (CEE), medroxyprogesterone acetate (MPA), CEE + MPA and tamoxifen ($n = 5$ in all groups). The immunohistochemical expression of PRA, PRB and the androgen receptor (AR) in breast tissue was quantified by image analysis. Over all, the total PR immunostaining in glandular epithelium was more abundant during CEE (mean 12%) and tamoxifen (11%) treatment as compared to CEE/MPA (5%), MPA (4%) and untreated controls (6%). Differences in PRB expression were observed between treatment groups ($p < 0.05$). In the CEE group levels of PRA were unchanged while there was a decline in the CEE/MPA group. The mean PRA/PRB ratio in the CEE group was 2.7 and in the CEE/MPA group 0.2. Treatment with tamoxifen had effects similar to those of estrogen. There was in all groups a weak positive nuclear AR immunostaining. This is the first *in vivo* study on the effects on long-term hormonal treatment on the expression of PR isoforms in normal primate breast tissue. The results suggest that hormonal treatments have a different influence on the PRA/PRB balance in the breast.

Introduction

The progesterone receptor (PR) is expressed in two protein isoforms, PRA and PRB. The different proteins are transcribed from two different promoters that are independently regulated and may have different physiological functions [1–3]. PRB appears to be the transcriptional activator of progesterone responsive genes whereas PRA inhibits PRB and also estrogen receptor-dependent gene activation [1, 4, 5]. Absolute levels of PR as well as the ratio PRA/PRB has been shown to vary considerably according to hormonal status, for example, in the endometrium during the menstrual cycle and also in human breast cancer tissue. The ratio PRA/PRB may define the physiologic and pharmacologic response to progesterone in reproductive tissues [6, 7]. At present there is no information on the effects of different hormonal treatments on the expression of the two PR isoforms in normal breast tissue.

Combined estrogen/progestogen treatment for hormonal contraception and postmenopausal replacement (HRT) has been associated with an increased risk of breast cancer [8, 9] While estrogen is a known mitogen in breast epithelium, the role of progesterone is complex and incompletely understood. In many target organs, for example, the endometrium estrogens increase cell proliferation as well as PR mRNA

and protein levels [10]. These effects are antagonized by progesterone and it is well established that the risk of hyperproliferation and endometrial cancer following unopposed estrogen can be counteracted by progestogen addition [11, 12].

In contrast to what is seen in the endometrium, progestogen addition does not seem to have an antiproliferative, protective effect in the breast. In fact combined estrogen/progestogen treatment may carry a risk for breast cancer beyond that of estrogen alone [13].

Little is known about the function of the androgen receptor (AR) in human breast tissue. The expression of AR in primary breast cancers suggests that this receptor may be involved in the response to endocrine treatments [14, 15]. PR and AR are members of the nuclear receptor super family. Synthetic progestogens have a high affinity for PR but also bind with a lower affinity to AR [16]. In women using oral contraceptives we recently demonstrated an association between circulating levels of both progestogen and androgens and proliferation in breast epithelium [17].

Studies on the effects of long-term hormonal treatment on the breast are difficult to perform in women. As shown in several studies cynomolgus macaques provide a relevant model to explore hormonal treatment effects on breast and endometrium [18]. In general experimental findings in macaques have been predictive of outcomes in human reproductive studies.

Materials and methods

Animals

The subjects of this study were 25 feral adult female cynomolgus macaques (*Macaca fascicularis*) imported from Indonesia to the United States (Charles River Primates, Port Washington, NY). They were 4–6 years of age at entry into the study. Animals were housed in social groups of four to eight monkeys each in a facility accredited by the Association for the Advancement and Accreditation of Laboratory Animal Care. Experimental protocols were approved by the Institutional Animal Care and Use Committee. Bilateral ovariectomies were done on all animals 3 months before the start of hormone treatment. They were treated with either 0.625 mg/day conjugated equine estrogens (CEE); 2.5 mg/day medroxyprogesterone acetate (MPA); CEE + MPA in continuous combination or 20 mg/day tamoxifen (TAM) ($n = 5$ in all groups). Test compounds were administered in the diet for 35 months at doses equivalent on a caloric basis to those given to women [19].

Tissue collection

Breast tissues were collected at the end of the treatment period, when all monkeys were euthanized and necropsied. Tissues were fixed in 4% paraformaldehyde for 24 h and stored at 4°C in 70% ethanol. Thereafter tissues were trimmed to 3 mm in thickness and embedded in paraffin.

Immunohistochemistry

Paraffin sections (5 μm) were used and a standard immunohistochemical technique (avidin–biotin-peroxidase) for immunolocalization of PRAB, PRB and AR was carried out as described previously [20]. After the tissue sections were dewaxed and rehydrated, an antigen retrieval procedure was performed. Pretreatment was performed by heating sections in a microwave oven at 700 W in 0.01 M sodium citrate buffer (pH 6.0). Non-specific endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide. Before incubation with the primary antibody all tissue sections were exposed to a non-immune block with normal horse serum. Different immunostaining procedures for each specific antibody were performed as follows:

PRAB. A monoclonal mouse anti-human antibody was used for detection of PRA + B (18–172, Zymed Laboratories, Inc., San Francisco, CA). The antibody recognizing the N-terminal of human PRA and B was diluted 1:150 and incubated for 30 min at room temperature. The secondary antibody (biotinylated horse anti-mouse) was added and incubated for 30 min, followed by an avidin–biotin-peroxidase complex treatment for 30 min at room temperature.

PRB. A monoclonal mouse anti-chicken antibody was used for the PRB immunostaining (MA1-411, Affinity Bioreagents, Inc., Golden, CO). The antibody reacts with the 120 kDa B isoform of human PR. The antibody was diluted 1:50 and incubated at room temperature for 1 h. The secondary antibody (biotinylated horse anti-mouse) was added and incubated for 1 h and followed by an avidin–biotin-peroxidase complex treatment for 30 min at room temperature.

PRA. PRA values were estimated by calculating the difference between PRAB and PRB immunostaining.

AR. A monoclonal mouse anti-human antibody (NCL-AR-2F12, Novocastra, UK) was used for detection of AR. The primary antibody recognizes the N-terminal domain of the AR and was diluted 1:25 and incubated overnight at 4°C. The procedures for the secondary antibody and avidin–biotin-peroxidase complex were the same as for the PRAB immunostaining.

The peroxidase substrate diaminobenzidine (DAB) was used to visualize the reaction (SK 4100; Vector Laboratory, Burlingame, CA, USA) for all the immunostaining. Thereafter the procedure was as described before [20].

Image analysis

A Leica microscope and Sony video camera (Park Ridge, NJ, USA) connected to a computer using an image analysis system (Leica imaging system Ltd., Cambridge, UK) were used to assess semiquantitative values from immunohistochemistry. The quantification of immunostaining was performed as described previously [21]. In short, by using color discrimination software the total area of positively stained nuclei was measured by an observer blinded to treatment, and expressed as the ratio of the total area of cell nuclei. The variation from 10 observations was 2%.

Immunohistochemistry scoring

For AR immunostaining intensity, scores $(+/-) = 1$, $(+) = 2$, $(++) = 3$, $(+++) = 4$ were determined by two independent observers blinded to treatment. The intraobserver correlation was $r_s = 0.83$, $p = 0.0002$. Scores were expressed as the mean value from the two observers.

Statistics

Differences between PRAB and PRB in the total material were assessed by the Wilcoxon Signed Rank test and correlations with the Spearman Rank correlation test. Differences between groups were assessed by ANOVA-on-ranks (Kruskal–Wallis) and the Mann–Whitney *U*-test. A p-value of <0.05 was considered as significant.

Results

Total PR and PRB were both expressed in the nuclei of macaque breast cells (Figure 1). Overall, the total PR immunostaining in the glandular epithelium was more abundant during CEE (mean 12%) and tamoxifen (11%) treatment as compared to CEE/MPA (5%), MPA (4%) and untreated controls (6%). The mean percentage area of cells positive for PRA in the untreated control group was 5% and for PRB 1% (mean PRA/PRB ratio = 4.8).

Differences in PRB expression were observed between treatment groups ($p < 0.05$). In animals treated with CEE, CEE/MPA and tamoxifen there was an increase in the expression of PRB (mean 6%) as compared to controls (1%). In the CEE group levels of PRA were virtually unchanged (6 and 5%, respectively). The mean PRA/PRB ratio in the CEE-group was 2.7. In contrast, in the CEE/MPA group, there was a numerical decline in PRA values (mean 1%) as compared to the control group (5%). The mean PRA value was the lowest among all groups and the mean PRA/PRB ratio in the CEE/MPA-group was 0.2.

Treatment with tamoxifen had effects quite similar to those of estrogen with an increase in PRB to 6% but no apparent change in PRA was observed.

During treatment with MPA alone there was no change in either PRA or PRB levels. There was, in all treatment groups, a positive nuclear AR immunostaining in the epithelial cells of the glands (Figure 1). Overall, the immunostaining intensity was weak and most slides were scored as 0 to ++. There were no apparent correlations with PR expression or differences between groups.

Discussion

This is the first *in vivo* study on the effects of long-term hormonal treatment on the expression of PRB and the PRA/PRB balance in normal primate breast tissue. Macaques have well-documented similarities to women in terms of reproductive physiology and anatomy, peripheral steroid hormone metabolism, mammary gland development and sex steroid receptors [18]. The epithelial cells also share a characteristic cytokeratin phenotype which differs from that in rodents and reflects the close phylogenetic relation between the species [22]. The percentage area of cells found positive for PR immunostaining in the present study is in agreement with previous data. According to hormonal status about 5–15% of breast epithelial cells were stained positive for PR in both monkeys [23, 24] and women [25, 26].

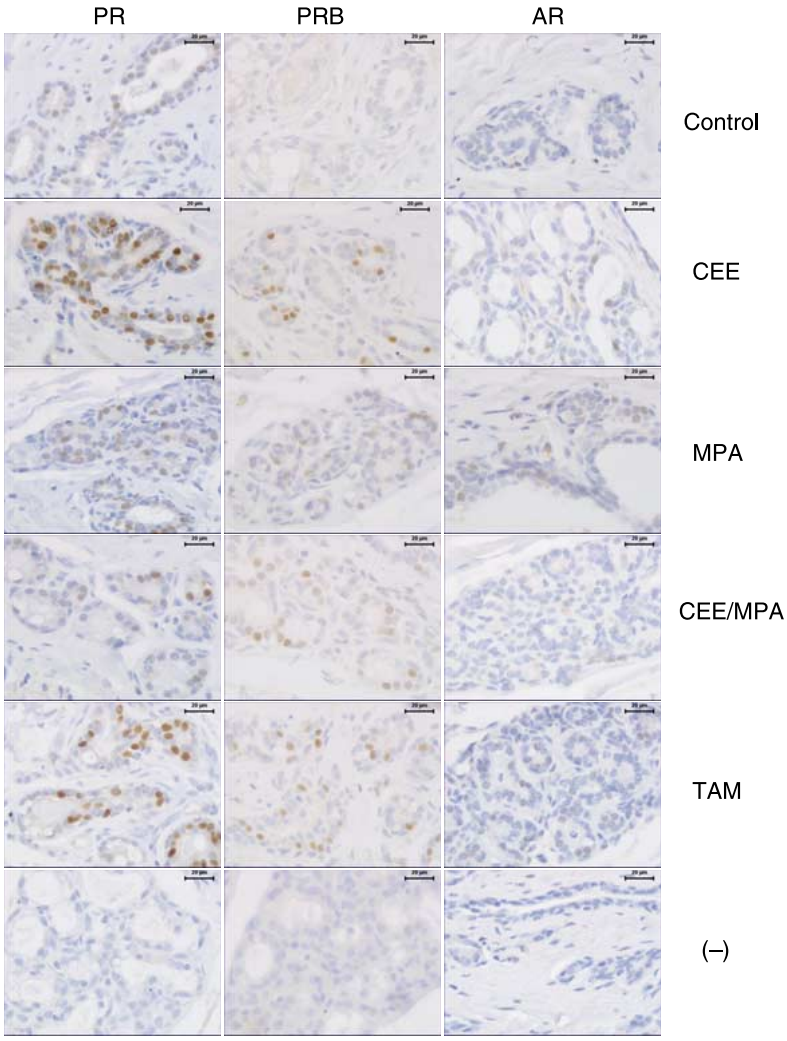

Figure 1. Representative pictures of immunostaining for total PR, PRB and AR in the different study groups. CEE: conjugated equine estrogens; MPA: medroxyprogesteroneacetate; CEE/MPA: a combination of conjugated equine estrogens and medroxy progesteroneacetate; TAM: tamoxifen, and control; (−): negative control.

As expected there was an increase in total PR during treatment with CEE. Estrogen is the major physiologic modulator of PR levels in reproductive tissues and may also influence the PRA/PRB balance [6, 27]. Here the increase in total PR was mediated by a rise in PRB while PRA levels seemed unchanged relative to controls. In breast cancer cells, estrogen has previously been found to increase PR expression via PRB and variations in A/B ratio have been reported in human breast cancers [7, 28].

In the breast progestogens appear to have both stimulatory and inhibitory effects. Whether alterations in the PRA/PRB ratio may mediate different effects, for example, on enzymatic metabolism proliferation rate and susceptibility to carcinogenesis in breast cells is unknown. In MCF-7 breast cancer cells PRA mediates antiestrogenic effects on endogenus estrogen receptor (ER) activity [29]. A differential effect of the two PR isoforms on estradiol-dependent transcription has been reported and PRB initiated ER activation [30]. Available data indicate that PRA and PRB are functionally different and PRA has been shown to act as a dominant repressor of PRB function [1, 4, 5].

There is accumulating evidence that there may be important differences between different hormonal treatment regimens regarding the effects on the breast. Recent studies in women using HRT have found a combination of estrogen and progestogen to be

associated with a higher risk of breast cancer than treatment with estrogen alone [13]. The present exploratory pilot study was not designed to assess proliferation. However, previously using the same macaque model and also in women, proliferation of breast epithelial tissue has been shown to be more pronounced during combined estrogen/progestogen HRT [23, 31]. Increased breast density may be a marker of an increased breast cancer risk and it has been demonstrated that more women using combined estrogen/progestogen treatment react with increased mammographic density than those on estrogen only treatment [32, 33].

In the present study we elevated long-term effects in the macaque model. After 35 months of treatment with CEE in combination with MPA the levels of PRB displayed the same significant increase as in animals treated with CEE only. This is in good agreement with findings in postmenopausal women [31]. In contrast no effects of treatment with MPA alone on either PRA or PRB were observed. However in the CEE/MPA group we also found indications for a simultaneous suppression of PRA expression (Figure 2). The mean percentage area of positive cells was only 1% although not significantly different from the 5% in the control group. The material in this study was limited and certainly the result must be interpreted with caution. As in previous studies of the breast both in the human and in non-human primates there was an interindividual variation in the response to exogenous hormones [17, 18, 23, 34]. The mean PRA/PRB ratio of 4.8 in the control group was reduced to 2.7 by CEE and was only 0.2 in animals receiving CEE/MPA. It is tempting to speculate that these apparent changes in the balance between the two PR isoforms could be relevant for differences in the cellular response to hormonal treatments.

We found tamoxifen to have effects similar to estrogen with an increase in PRB and no change in PRA levels. Tamoxifen is widely used in the treatment of breast cancer, acting as an estrogen antagonist with antiproliferative effects. However in other target organs such as bone, endometrium and vaginal epithelium it also has estrogenic properties [35]. The results confirm our previous findings in the macaque model that the divergent agonistic/antagonistic effects of tamoxifen are not limited to a between organ variation but are also present in the breast [23, 36]. Thus there is an apparent increase in PR expression which should be regarded an estrogen-like effect which is not accompanied by an increase proliferation.

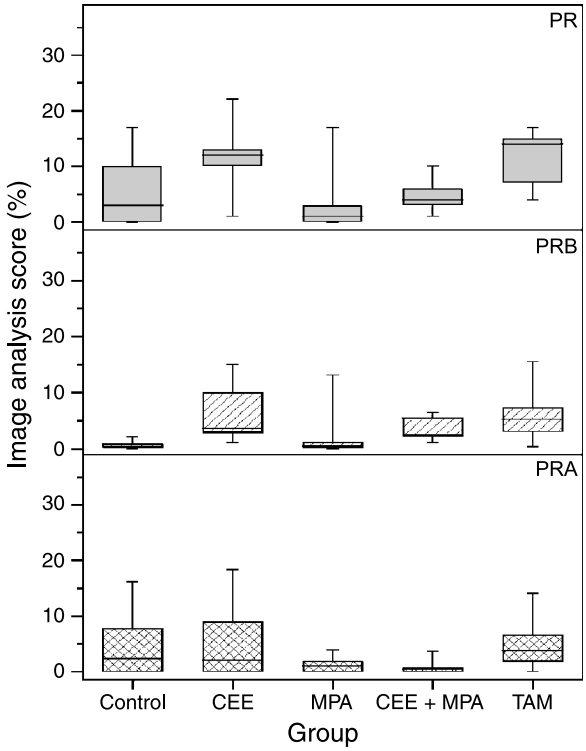

Figure 2. Box-and-whisker plots representing the median value with 50% of all data falling within the box. The 'whiskers' extend to the fifth and 95th percentiles. Percentage area of cells positive for total PR, PRB and PRA in controls and different study groups. CEE: conjugated equine estrogens; MPA: medroxyprogesterone acetate; CEE/MPA: a combination of CEE and medroxyprogesterone acetate; TAM: tamoxifen, and controls ($n = 5$ in all groups).

In the present small material we found no significant differences in AR expression according to treatment groups. Only few positive cells were observed in the samples from each group and in fact the most intensive staining was observed in apocrine glandular epithelium (data not shown). Still a predictive value of AR expression has been suggested in women with advanced breast cancer [14]. Clearly further studies and larger materials are needed to confirm or exclude any effects of exogenous estrogen and progestogen on AR expression in the normal breast.

Female sex steroids are mitogens in breast tissue and the balance between the effects of estrogen and progestogen are of particular importance. The two PR isoforms A and B may have different roles to modulate the cellular response following ER activation. We have demonstrated that different hormonal treatments may have a different impact on PRA and PRB expression in the breast and thus on the PRA/PRB balance. While the results need further confirmation they may

reflect mechanisms of importance for the regulation of hormonal breast response and thus be relevant for the different effects and risks of different hormonal treatments.

Acknowledgements

The expert technical assistance by Torsten Hägerström and Catharina Karlsson is gratefully acknowledged. This study was supported by grants from the Swedish Medical Research Council (grants 3972 and 5982), the Cancer Society in Stockholm, Magn. Bergvalls foundation, the Swedish Society for Medical Research, Karolinska Institutet; grant DAMD17-94-J-4201, US Army Medical Research Acquisition Activity, Fort Detrick, MD and grants HL-490852 and HL-45666, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD.

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