New insight on a possible mechanism of progestogens in terms of breast cancer risk

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Abstract

Objectives: Progestogens influence mammary gland development and probably breast cancer tumorigenesis by regulating a broad spectrum of physiological processes. We investigated receptor membrane-initiated actions of progestogens in MCF-7 breast cancer cells overexpressing progesterone receptor membrane component 1 (PGRMC1).

Design: MCF-7 cells were stably transfected with PGRMC1 expression plasmid (MCF-7/PGRMC1-3HA) and overexpression of PGRMC1 was verified by immune fluorescent analysis and Western blot. To test the effects of progestogens on cell proliferation, MCF-7 and MCF-7/PGRMC1-3HA cells were stimulated with a membrane-impermeable progesterone: BSA-fluorescein-isothiocyanate conjugate (P4-BSA-FITC), unconjugated progesterone (P4), medroxyprogesterone acetate (MPA), norethisterone (NET) and drospirenone (DRSP). Furthermore, reverse phase protein technology was applied to identify modified downstream signaling.

Results: Progesterone did not elicit any proliferative effect on MCF-7/PGRMC1-3HA cells. By contrast, P4-BSA-FITC, DRSP, MPA and NET significantly triggered proliferation of MCF-7/PGRMC1-3HA cells, the effect being more pronounced for NET. Almost no effect of progestogens on proliferation was observed in MCF-7 cells. In MCF-7/PGRMC1-3HA cells, expression of Erk1/2 was significantly reduced by 40% compared to MCF-7 cells.

Conclusions: Our data indicate that PGRMC1 mediates a progestogen-dependent proliferative signal in MCF-7 cells. Of significant interest is that progesterone and synthetic progestins that are used for hormone therapy are different in their proliferative effects on MCF-7 and MCF-7/PGRMC1-3HA cells. Progesterone appears to act neutrally, whereas MPA, NET and DRSP trigger proliferation and thus might increase breast cancer risk. The data presented are very important in terms of the positive results of progestogens and breast cancer risk in clinical studies so far.

Keywords: progestogens; progesterone receptor membrane component 1; proliferation; signal cascade.

Introduction

Hormone therapy and oral contraception are still an important risk factor for breast cancer. Evidence is accumulating that progestogens can play a crucial role. Progesterone receptor membrane component 1 (PGRMC1) is expressed in breast cancer [1, 2]. It could be important in tumorigenesis and thus could increase breast cancer risk. Little is known about downstream signaling pathways [3, 4].

The role of progestogen addition to estrogen therapy in postmenopause has come under scrutiny since the results of the Women’s Health Initiative (WHI) mono arm were published compared to the WHI combined arm [5, 6]. The WHI trial used the combination of conjugated equine estrogens plus medroxyprogesterone acetate (MPA). In contrast to the WHI combined arm, in the estrogen only arm no increase but rather a reduction of breast cancer risk was found, which was significant for patients with more than 80% adherence to study medication [7]. This result indicates a negative effect of progestogens with regard to breast cancer risk. However, it remains unclear whether the combination of estrogens with synthetic progestins and/or natural progesterone can elicit the same increased risk. Thus, many questions concerning the extrapolation of the WHI results to all synthetic progestogens and to natural progesterone remain unanswered. Of special note is one cohort study using micronized progesterone in combination with estrogens, which detected no increase in breast cancer risk when combining transdermal (patches) or percutaneous (gels) estradiol therapy with progesterone [8]. In 80,377 women breast cancer risk was increased with oral synthetic progestogens, but not with progesterone and dydrogesterone. The mean duration of hormone therapy (HT) was 7 years. This study had a mean follow-up of 8.1 years.

Progestogens are conventionally thought to act via the activation of the intracellularly located progesterone receptors (PRs), PR-A and PR-B. Several in vitro studies indicate that progestogens can exert an antiproliferative effect by activation of these receptors in human breast cancer cells [9–11].
These data are in contrast to the above mentioned clinical data. Other data suggested a proliferative effect of synthetic progestogens [12, 13]. Thus, the mechanisms by which progestogens act on human breast cells remain unclear.

Recent experimental data revealed that in addition to the intracellular-located receptors, PGRMC1 is associated with a membrane-associated progesterone receptor activity [3]. PGRMC1 was originally cloned from the endoplasmatic reticulum from porcine hepatocytes [14]. It contains several predicted motifs for protein interactions and overlapping sites for phosphorylation, for which phosphorylation status might correlate with its localization in the cell [3, 15, 16]. PGRMC1 has been detected in several cancers and cancer cell lines, e.g., breast cancer [2, 17]. It is overexpressed in lung cancer and colon cancer [3].

There is a long-standing link between PGRMC1 and progesterone signaling. However, because bacterially expressed PGRMC1 does not bind to progesterone [18], and because the majority of PGRMC1 is not localized to the plasma membrane [1, 19, 20] it is now tentatively assumed that PGRMC1 does not bind P4 by itself [3], but requires an unknown protein that is associated only in partially purified PGRMC1 preparations [21]. PGRMC1-associated progesterone binding is functionally important in cancer cells because progesterone inhibits apoptosis in granulosa cells, and this antiapoptotic activity requires PGRMC1 [21, 22]. However, it is unclear how PGRMC1 transduces antiapoptotic signaling by progesterone. Expression of PGRMC1 has been identified in several subcellular compartments including cell membrane, cytoplasm, endoplasmatic reticulum and nucleus (reviewed in [3]). Swiatek-De Lange et al. reported that PGRMC1 localizes to the plasma membrane and microsomal fraction of retinal cells [23]. Based on these data in this study, we investigated receptor membrane-initiated actions of progesterone and various synthetic progestins in MCF-7 breast cancer cells overexpressing PGRMC1 as well as the influence of the overexpression of PGRMC1 on the activation of important signaling proteins.

Methods

Cell cultures

MCF-7, a human estrogen receptor (ER) positive primary breast cancer cell line, was purchased from American Type Culture Collection (ATCC). Cells were routinely cultured in RPMI-1640 medium containing 10% (v/v) heat inactivated fetal calf serum, 25 mM HEPES and 1% penicillin/streptomycin at 37°C in a humid 5% CO₂ atmosphere.

Transfection of MCF-7 cells

MCF-7 cells were stably transfected with expression vector pCDNA3.1 containing heme agglutinin-tagged (3HA) PGRMC1 using lipofectamine™ 2000 (Invitrogen, Karlsruhe, Germany), in accordance with the manufacturer’s recommendation. A total of 5 x 10⁶ cells were transfected and plated with RPMI medium for 24 h. Then, the medium was changed to RPMI complete medium containing 100 µg/mL hygromycin B. Cells were cultured for 2 weeks for selection of stable integration events. Transfection rates were measured by cotransfection of a GFP-expressing plasmid and immune fluorescence analysis. After 2 weeks single colonies had formed and limiting dilutions were performed three times to select for colonies grown from a single cell.

Stable transfection was verified by PCR using chromosomal DNA and primers spanning intron 1 to distinguish integrated PGRMC1 cDNA from the chromosomal sequence. The sequences of the primers were 5'-CTGCTGCATGAGATTTTCGAC-3' hybridizing to nucleotides 71–91 of PGRMC1 open reading frame and 5'-GCATAGTCGAGACGTCA-3' hybridizing to the sequence coding for the HA tag. PCR products were sequenced.

Dissolution of progestins

Progesterone (P4), MPA, norethisterone (NET) and progesterone-3-(O-carboxymethyl) oxime: BSA-fluorescein-isothiocyanate conjugate (P4-BSA-FITC) were purchased from Sigma (Munich, Germany) and dropsoiren (DRSP) from Schering (Berlin, Germany). P4-BSA-FITC was dissolved in H₂O and stored as 10⁻⁴ M at 4°C for less than 1 week, whereas the other compounds were dissolved in ethanol, stored as 10⁻² M at −20°C and further diluted during experiments to a final ethanol concentration of less than 0.01%.

Cell proliferation

All proliferation assays were conducted using stripped medium, i.e., phenol red-free medium with charcoal/dextran-treated fetal bovine serum (Thermo, Karlsruhe, Germany). Approximately 10,000 cells were seeded in normal medium per well in 96-well plates. On the next day the medium was changed to stripped medium and the cells were incubated at 37°C for 2 days. Afterwards, stripped medium containing progestogens at concentrations ranging from 10⁻⁶ M to 10⁻¹⁰ M was added. Cell proliferation was measured by MTT assay after 4 days of incubation as described earlier [17]. In brief, yellow MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole] was reduced to purple formazan in the mitochondria of living cells. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by a spectrophotometer. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. In in-house experiments we have validated the MTT assay against the BrdU assay.

For kinetic studies the same cell lines were incubated with all progestogens mentioned above at 10⁻⁶ M for 6 days. Medium was changed every 2 days and proliferation was measured daily by the MTT assay.

Reverse phase protein microarrays (RPPMs)

Protein expression profiling was performed using RPPMs and the planar waveguide-based ZeptoMARK platform (Zeptosens, Witterswil, Switzerland) [24]. For analysis, flash frozen cell pellets were lysed. Using the NanoPlotter 2 (GeSim, Grosserkmannsdorf, Germany) samples (400 pl) were spotted onto ZeptoMARK Protein Microarray Chips (Zeptosens). On each chip, six identical arrays each containing 80 samples in quadruplicate were generated. After spotting, the microarrays were blocked with 3% BSA, washed thoroughly with double distilled H₂O, dried in a stream of nitrogen and stored in the dark at +4°C until use. The immobilized protein content of a given spot in a microarray was determined by protein staining with a reactive fluorescent dye that binds to primary amines.
of the immobilized proteins (Alexa647-NHS-ester; Invitrogen, Carlsbad, CA, USA) according to manufacturer’s protocol for labeling proteins. Detection of proteins was performed using a direct two-step immunoassay using diluted primary antibody and fluorescence-labeled anti-species secondary antibodies in assay buffer. Blank incubations (i.e., assays with no primary antibody) were performed under identical conditions. After washing images of the microarrays were taken using the ZeptoREADER® imager (Zeptosens) and analyzed using the array analysis software ZeptoVIEW™ Pro 2.0 (Zeptosens). Signal intensity for each spot was determined as background-corrected mean intensity with the local background subtracted from the spot intensity. Each of the measured values was referenced against adjacent reference spots as implemented in the software package giving referenced fluorescence intensity (RFI) units that are independent on image exposure time.

Fluorescence signals (RFI) for four replicate spots were averaged and the blank signal determined for these spots was subtracted to correct the assay signals for the contribution of non-specific binding of the secondary antibody, yielding blank-corrected MFI signals. In a final step, the signal intensity was normalized against the protein content in the spot by using the signal intensities from the on-chip protein determination. Therefore, the calculated signal intensities correspond to normalized, blank-corrected MFI values (NFIs). Standard deviations were calculated according to standard error propagation rules from the standard deviations of raw, blank and protein stain signals. NFI values were used for all subsequent statistical analysis.

**Western blot analysis**

Cells were washed twice with ice-cold phosphate buffered saline (PBS) and lysed in M-PER mammalian protein extraction reagent containing Halt Protease Inhibitor Cocktail according to the manufacturer’s protocol (both from Pierce, Rockford, IL, USA). Protein concentrations were determined using the BCA Protein Assay Kit (Pierce). In total, 25 μg of protein extract was loaded per lane onto a 10% polyacrylamide gel and separated by electrophoresis. The gel was blotted onto a Hybond ECL nitrocellulose membrane (Amersham, Piscataway, NJ, USA) at 15 V for 90 min using a semi-dry blot system. The membrane was blocked for 2 h at room temperature using 5% dried low fat milk powder dissolved in TBST buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20). Then, the first antibody was incubated overnight at 4°C. After washing three times with TBST, the second antibody was incubated for 2.5 h at room temperature. Chemiluminescence was generated using the ECL Western Blotting Analysis System (Amersham). The signals were measured and quantified with a Lumi-Imager and LumiAnalyst 3.1 software (Boehringer, Mannheim, Germany). Western blot analysis was used to measure PGRMC1 and Erk1/2 expression. The respective first antibodies were: rabbit PGRMC1 antibody (G21, sc-133906, 1:200, Santa Cruz, CA, USA) and rabbit Erk1/2 polyclonal antibody (1:10000) (Cell Signaling Technology, Danvers, MA, USA). Goat anti-rabbit horseradish peroxidase (HRP, 1:1000, Santa Cruz) was used as secondary antibody.

**Preparation of cytospins**

For preparation of cytospins, 5×10⁴ cells were resuspended in 500 μL PBS spun onto slides using Cytospin 2 centrifuge (Shandon, Waltham, MA, USA) at 1000 rpm (130×g) for 5 min. Then, the cytospins were dried overnight at room temperature and stored at −20°C until further use. Cytospins were prepared as described earlier [17].

**Immune fluorescence analysis**

Immune fluorescence analysis was performed as described earlier [17]. Rabbit anti-HA (1:100) (Santa Cruz) was used as primary antibody and goat anti-rabbit AlexaFluor 594 (1:100, Invitrogen, Karlsruhe, Germany) was used as secondary antibody.

**Statistical analysis**

All proliferation experiments were done in triplicate and were repeated at least three times, with each experiment yielding essentially identical results. Statistical analysis was done by analysis of variance with the logarithmized values followed by Dunnett’s procedure from triplicates of at least three independent experiments. The overall α level was set at 0.05.

**Results**

**Transfection of MCF-7 cells with PGRMC1 coding expression plasmid**

Transfection rates measured by cotransfection of a GFP-expressing plasmid were around 40%–50% (data not shown). After subcloning by limiting dilution technology, PGRMC1-3×HA transfected MCF-7 cells were prepared for cytospins and immune fluorescence analysis was performed using anti-HA antibody indicating that the transfected cell line was almost devoid of non-transfected MCF-7 cells (Figure 1A).

Western blot analysis for the PGRMC1-3HA fusion protein resulted in a single band at approximately 30 kDa, which is the predicted size of 28 kDa for PGRMC1 plus approximately 3 kDa for the three HA tags (Figure 1B). The untransfected MCF-7 cells produce only a very faint signal at 28 kDa indicating a weak intrinsic PGRMC1 expression. Preincubation of the PGRMC1-specific antibody with synthetic PGRMC1 protein abolished both the exogenously and the endogenously PGRMC1 signal, confirming the specificity of the antibody (Figure 1B). Similar signal intensities for the housekeeping protein actin (approximately 42 kDa) indicate loading of equal amounts of total protein. PCR products for PGRMC1 from genomic DNA of the transfected cell lines provided the expected signal in the agarose gel (data not shown). Sequencing of PCR products confirmed the PGRMC1 wild-type sequence. At the end of the experiments the high purity of the MFC-7/PGRMC1-3HA cells was validated by immune fluorescence analysis of cytospins and by sequencing PCR products. These results indicated that MFC-7/PGRMC1-3HA cells are highly pure, overexpress PGRMC1 wild-type protein and can be used for functional assays.

**Exploration of signal proteins**

To explore signal proteins regulated by PGRMC1 expression or proteins potentially involved in downstream PGRMC1 signaling, reverse phase protein array technology was applied. With that purpose, MCF-7 and MCF-7/PGRMC1-3HA cells were stimulated with P4, P4-BSA-FITC, MPA and NET and the expression levels of 15 signal transduction proteins were determined: Akt, Bad, Bax, c-Fos, c-Jun, Caspase...
3, Cyclin D1, Cyclin E2, Erk1/2, IκB, Ki67, mTOR, PCNA, PI3K and PKC.

Because there are only scarce data about potential downstream signaling pathways, proteins were selected based on published data [IκB, Akt [25], PI3K, PKC, Erk1/2 [23], Bad [26]] and based on its potential involvement in apoptosis and proliferation.

Stimulation did not change the expression levels of these proteins in four replicate experiments. Only the basal expression, i.e., without stimulation, of Erk1/2 protein was significantly downregulated by approximately 40% in MCF-7/PGRMC1-3HA cells compared to MCF-7 cells (Figure 2A). This result was confirmed by Western blot analysis using independent cell lysates (Figure 2B).

**Synthetic progestins increase proliferation of PGRMC1 overexpressing MCF-7 cells**

Because Erk1/2 are members of the mitogen-activated protein kinase superfamily that can mediate cell proliferation, we first investigated potential differences in the basal proliferation of MCF-7 and MCF-7/PGRMC1-3HA cells. The latter showed a significant higher proliferation after day 7 (Figure 3A). Dose-dependent effects on cell proliferation of P4, P4-BSA-FITC, MPA, NET or DRSP were then determined using the MTT assay (Figure 3B). Between 10−9 M and 10−5 M P4 did not increase proliferation of either MCF-7 or MCF-7/PGRMC1-3HA cells. However, proliferation of MCF-7/PGRMC1-3HA cells was significantly increased when treated with P4-BSA-FITC or the synthetic progestogens: for P4-BSA-FITC at concentrations from 10−7 M to 10−5 M with a maximal effect at 10−6 M, for NET reaching its maximal effect compared to untreated control at 10−7 M, for MPA at concentrations higher than 10−6 M, and for DRSP at concentrations higher than 10−7 M. The effect of NET was significantly different to that of DRSP at the concentrations of 10−9 and 10−8 M and to the effect of MPA at the concentrations of 10−9, 10−8 and 10−7 M. DRSP showed a significantly stronger effect compared to MPA at the concentration of 10−7 M.

The clinically relevant blood concentrations for the progestogens most commonly used for HT, MPA and NET are in the range of 4×10−9 M to 10−8 M for MPA [27] and around 10−8 M for NET [28]. However, higher concentrations might be required in vitro in short-time tests in which the reaction threshold can only be achieved with supraphysiological dosages. Higher concentrations can also be reached in vivo in the vessel wall or organs compared to the concentrations usually measured in the blood.

No effects were observed in MCF-7 cells within the investigated concentration ranges for all the progestogens used in this experiment. For further kinetic experiments, 10−6 M was chosen for all progestogens. In comparison to all other synthetic progestogens tested, NET significantly increased proliferation almost to maximum even at 10−9 M, the lowest concentration that we tested. Taken together, the results strongly suggested that some progestogens elicit a PGRMC1-dependent proliferative response.

To determine time-dependent proliferative effects of progestogens, a kinetic analysis over 6 days was performed (Figure 3C). MCF-7 and MCF-7/PGRMC1-3HA cells were incubated with P4, P4-BSA-FITC, DRSP, MPA and NET at 10−7 M and proliferation was determined by MTT assay. The results indicate that P4-BSA-FITC, DRSP, MPA and NET
FITC, DRSP, MPA and NET at 10^{-6} M. Cell proliferation was measured after 4 days. Data were normalized to unstimulated controls (means ± SD). **p < 0.01 vs. controls).

**Discussion**

Hormones, acting through their receptors, drive the proliferation of several tumor types, including breast, ovarian and prostate tumors. Progesterone-regulated responses are mediated by an array of progesterone receptors that include the classic nuclear PR-A and PR-B receptors and splice variants of each. During the past years, evidence has accumulated that at least three PR groups exist which could be potentially involved in membrane-activated progesterone signaling: (a) intracellular PRs localizing at the plasma membrane, (b) PGRMC1, and (c) members of the newly described family of the G-protein-coupled seven transmembrane domain 7TMR family.

**Signal transduction of PGRMC1**

There are limited data concerning PGRMC1-activated signaling pathways. PGRMC1 plays a role in regulating protein kinase-associated signaling in which PGRMC1 increases Akt activation and IκB phosphorylation leading to NFκB activation [32]. Akt is phosphorylated by the PDK1 protein kinase, and there is a putative PDK1 binding region on PGRMC1. The exact mechanism through which PGRMC1 activates Akt is unknown. Because PGRMC1 has several potential binding sites for interacting proteins it might act as a type of adaptor protein, providing docking sites for proteins that activate Akt, such as PDK1. Membrane-impermeable progesterone conjugate induces calcium influx and subsequent phosphatidylinositol-3-kinase-mediated phosphorylation of PKC and Erk1/2 [23]. A putative Erk2 consensus site at T177 in PGRMC1 [31] has been demonstrated to be phosphorylated in vivo [33, 34]. Erk2 is induced by PGRMC1 and could therefore be involved in feedback inhibition of signaling through the adjacent putative SH2 protein interaction motif centered on the amino acids Y190 and S180. S180 appears to be constitutively phosphorylated in non-stimulated MCF-7 cells [18].
and is presumed to require the activation of a phosphatase for the interaction of putative SH2 domain proteins with Y179 [2]. Unlike PGRMC1 phosphorylated at Serine 180, tyrosine phosphorylated PGRMC1 is not located in the endoplasmic reticulum [3, 16].

The mechanisms of the proliferative effect observed in MCF-7/PGRMC1-3HA cells are currently unknown. Reverse protein array technology revealed that basal Erk1/2 levels appear to be reduced by approximately 40% in MCF-7/ PGRMC1-3HA cells compared to MCF-7 cells. Stimulation of cells with progestogens does not alter Erk1/2 expression. In rat neural progenitor cells, Liu et al. found that P4 induced a PGRMC1-dependent cell proliferation which required the Erk signaling pathway [34].

**Synthetic progestins increase proliferation of PGRMC1 overexpressing MCF-7 cells**

Since the results of the WHI mono arm were published, indicating a negative effect of progestins on breast cancer risk, the molecular pathway responsible for this effect and the many questions on the extrapolation of the WHI results to all synthetic progestins and to natural progesterone remain unknown. Here, we present the first results suggesting that signaling of synthetic progestins via PGRMC1 could be one explanation. We have chosen two synthetic progestins that are widely used in hormone therapy, i.e., MPA and NET, as well as a new synthetic progestin, i.e., DRSP, which might differ in its behavior to MPA and NET because of a different chemical structure.

The synthetic progestins MPA, NET and DRSP significantly induced a relatively large proliferative effect in MCF-7 cells that overexpress PGRMC1. For P4, however, no such effect was found. Because progesterone and the synthetic progestins used in HT are able to activate PR-A/-B and PGRMC1 simultaneously, our data suggest that in vivo the balance of the expression levels of both receptors might influence whether epithelial cells proliferate or not in the presence of progestogens. Therefore, it might be instructive to determine the expression ratio of PGRMC1 and PR-A/-B before HT.

Interestingly, P4-BSA-FITC is able to induce a marginal proliferative signal in MCF-7 cells (Figure 3B). P4-BSA-FITC is thought to be unable to cross the plasma membrane and can therefore only bind to membrane associated progesterone receptors. MCF-7 cells express endogenous PGRMC1 at very low amounts (Figure 1) which can transduce the weak proliferative signal because the classical PR-A/-B response is not triggered. The synthetic progestins and P4 bind to all progesterone receptors expressed by MCF-7 cells. Binding to PR-A/-B might transduce an antiproliferative signal, countering the proliferative signal induced by low levels of PGRMC1. By contrast, in MCF-7/PGRMC1-3 HA cells the exogenously expressed PGRMC1 might oppose the antiproliferative effect of PR-A/-B. In several human ovarian surface epithelial cell lines, P4 inhibits their proliferation [35]. Because these cells express PR-A/-B it has been assumed that the actions of P4 are mediated via these receptors. However, P4 exhibits antimitotic action only at micromolar doses, which have been used in these experiments [35]. Given that the dissociation constant of the progestins for the PR-A/-B is 1–5 nM [36] and for PGRMC1 is in the 0.20–0.3 μM range [14], which is well within the levels of P4 in serum and in follicular fluid [37], in MCF-7 cells the classical PR-A/-B receptors are perhaps activated preferentially by gestagens inducing an antiproliferative signal. This concept is supported by a previous observation that at micromolar doses P4 inhibits granulosa cell and spontaneously immortalized granulosa cell mitosis [38].

Interestingly, NET exerts its activity on proliferation already at the lowest concentration tested (10–9 M, Figure 3B), whereas DRSP and MPA increase proliferation only at higher concentrations (10–7 M and 10–6 M). This suggests that NET binds PGRMC1 with the highest affinity, followed by DRSP and MPA. Compared to PR-A/-B this is different because the latter binds MPA better than NET [39]. These results indicate that HT including NET might result in an increased risk for breast cancer development. Indeed, some studies in which norethisterone- or levonorgestrel-derived progestogens were continuously administered a significantly higher risk for breast cancer was observed than for continuously administered progestrone-derived progestogens [40, 41]. In one study, the use of norethisterone acetate was accompanied with a higher risk after 5 years of use (2.03, 1.88–2.18) than that of medroxyprogesterone acetate (1.64, 1.49–1.79) [41]. It is known that NET can be converted in vivo into ethinylestradiol [42]. With regard to this conversion influencing the observed NET effect is currently unknown and is under investigation.

Serum concentrations of progesterone during HT are in the range of 10–8 M to 10–7 M, i.e., concentrations which do in fact have an effect in our experiments. However, experimental investigations can only point to mechanism(s) and clearly cannot replace clinical studies. In agreement with our results, NET alone at 10–6 M has been found to stimulate the growth of ER positive MCF-7 and T47DA18 human breast cancer cells, but not ER negative MDA-MB-231, BT-20 and T47DC4 human breast cancer cells [43]. Varying results regarding the effects of MPA on breast cancer cells have been published, but it seems to induce a modest and statistically significant cell growth at 10–6 M in a specific subgroup of MCF-7 cells [8].

**Conclusion**

The effect of progestins on breast cancer tumorigenesis can depend on the specific progestin used for hormone therapy and the expression of PGRMC1, PR-A and PR-B in the target tissue. However, in terms of the clinical situation it remains unknown how uniformly PGRMC1 is expressed in the normal breast epithelial cells between patients. Thus, screening, which might be based on determining the expression ratio of PGRMC1 and PR in cells from nipple aspirate fluid, might be of interest to identify women who show an increased expression of PGRMC1 and who might thus be susceptible for breast cancer development under HT [44].
The data presented here are of significant importance in terms of progesterone and breast cancer risk in HT clinical studies so far [5, 6].

Acknowledgements

The authors thank B. Kootz and B. Pfister for technical support. M.A.C. was supported by a Research Fellowship from the Center for Inland Health, Charles Sturt University.

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