The Effect of Progesterone and Synthetic Progestins on Serum- and Estradiol-Stimulated Proliferation of Human Breast Cancer Cells

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Abstract

The results from the Women's Health Initiative study on enhanced breast cancer risk in postmenopausal women using an estrogen/progestin combination clearly indicate the need for a comparison of different progestins with regard to cancer risk. To shed some light on this issue, we have investigated the influence of progesterone and various synthetic C19- and C21-progestins on cell proliferation of a human breast cancer cell line *in vitro*. Of special interested was the comparison of two different regimens commonly used in HRT, sequential and continuous combination with estradiol. We used the human breast cancer cell line MCF-7 as a model. Progesterone (P), chlormadinone acetate (CMA), dienogest (DNG), gestodene (GSD), 3-ketodesogestrel (KDG), levonorgestrel (LNG), medroxyprogesterone acetate (MPA), and norethisterone (NET) were investigated in the range of 0.01 nm to 10 µM alone and in combination with 10 nM estradiol. Cell proliferation was measured after 7 days using the ATP chemosensitivity test. Tested alone, CMA, DNG, GSD, KDG, MPA and NET significantly stimulated cell proliferation, but only at high dosages. Sequentially combined with estradiol, only CMA inhibited cell proliferation over the whole concentration range. Slight effects were found for DNG, GSD and KDG, whereas P and MPA only showed an effect at the highest concentration. NET had no significant effect on cell proliferation. Continuously combined, all progestins exhibited an inhibitory effect over the whole concentration range. The most prominent effects were found for P, CMA, GSD, and KDG. Only slight effects were found for DNG, MPA and NET. Our in vitro results indicate that the influence on breast cancer risk using HRT in postmenopausal women might depend on the type of progestin used as well as on the regimen applied. However, the net inhibitory *in vitro* effect of the progestins at clinically relevant dosages is rather minimal, and whether progestins in general can reduce breast cancer risk in long-term treatment remains uncertain. Further clinical trials are urgently needed to clarify this issue.

Key words
Progestins · Combination with Estradiol · Proliferation · MCF-7 Cells

Introduction

Proliferation of human breast epithelial cells is clearly controlled by sex hormones; breast carcinogenesis seems to be the most serious side effect of hormone therapy [1]. The Women's Health Initiative (WHI) study was stopped after 5 years due to an increased incidence of breast cancer in the treatment group, indicating the great therapeutic concern of a possible association between hormone replacement therapy and breast cancer risk in postmenopausal women [2]. This was the first randomized prospective study to investigate the possible primary prevention of coronary heart diseases in postmenopausal women using a continuous combination of conjugated estrogens plus medroxyprogesterone acetate. However, the role of progestin addition, which is mandatory in women with an intact uterus to prevent endometrial carcinoma, in terms of breast cancer risk has been con-

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Received 30 July 2002 · **Accepted after revision** 8 October 2002

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Horm Metab Res 2003; 35: 76–80 © Georg Thieme Verlag Stuttgart · New York · ISSN 0018-5043

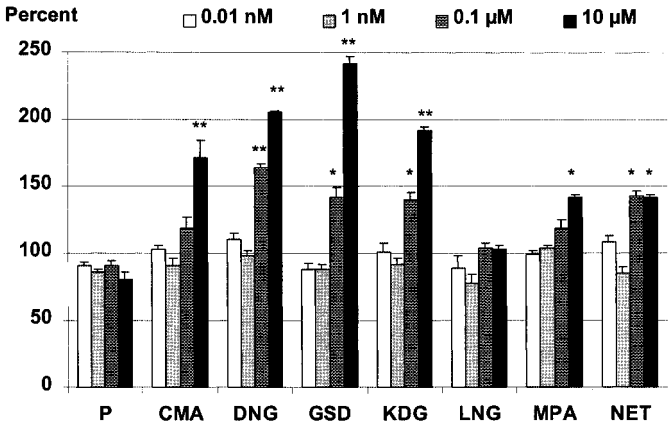

Fig. **1** Changes of serum-stimulated growth of MCF-7 cells after addition of progestins. P: progesterone, CMA: chlormadinone acetate, DNG: dienogest, GSD: gestodene, KDG: 3-ketodesogestrel, LNG: levonorgestrel, MPA: medroxyprogesterone acetate, NET: norethisterone (means ± SD, * $p < 0.05$, ** $p < 0.01$ vs. control value = 100 %).

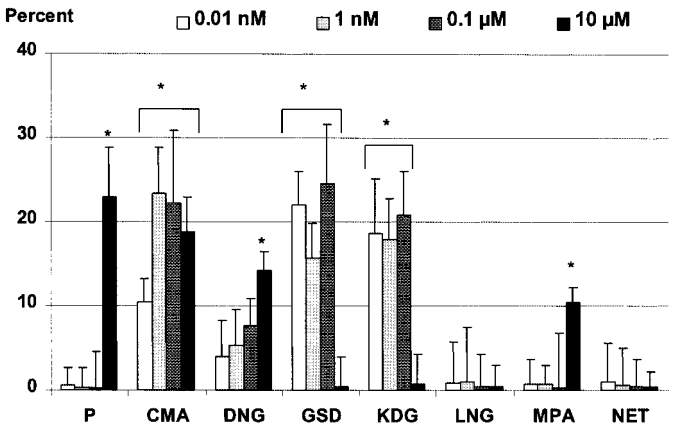

Fig. **2** Inhibition of estradiol (10^{-10} M) induced proliferation of MCF-7 cells by various progestins sequentially combined with estadiol. P: progesterone, CMA: chlormadinone acetate, DNG: dienogest, GSD: gestodene, KDG: 3-ketodesogestrel, LNG: levonorgestrel, MPA: medroxyprogesterone acetate, NET: norethisterone (means ± SD, * $p < 0.05$, $p < 0.01$ vs. estradiol value).

troversially discussed over the last few years. Two recent studies did not shed much light on this issue due to the low number of patients and lack of clarity on the kind of progestin used [3,4]. This problem exists for both main HRT regimens in current use – sequential and continuous combined progestin addition.

Therefore, to shed some light on this topic, we decided to investigate the effect of progesterone and seven synthetic progestins. These are already used for either HRT or contraception, or are currently subject to clinical study for their suitability in HRT regarding serum- or estradiol-stimulated proliferation of the estradiol receptor-positive breast cancer cell line MCF-7. The progestins were tested alone and in sequential and continuous combination with estradiol using physiological and pharmacological concentrations of the progestins. Although *in vitro* studies may not be easily extrapolated into *in vivo* conditions, these studies may help clarify the picture.

Material and Methods

Estradiol, progesterone, chlormadinone acetate, medroxyprogesterone acetate and norethisterone were purchased from Sigma Chemical. Gestodene, 3-ketodesogestrel and levonorgestrel were kindly provided by Wyeth Pharma, and dienogest by Jenapharm. The compounds were dissolved in ethanol and diluted by ethanol/PBS mixtures to yield a final ethanol concentration of less than 1 % per well.

MCF-7, a human estrogen and progestin receptor-positive breast cancer cell line, was purchased from ECACC, UK. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5 % (v/v) fetal calf serum supplemented with 0.3 mg/ml glutamine, 5 ng/ml bovine insulin and 100 U/ml penicillin plus 100 µg/ml streptomycin.

Ninety-six well plates were seeded at approximately 1000 MCF-7 per well in assay kit medium. After that, the cells were incubated with medium containing charcoal/dextran-treated serum for 3 days. The progestins were then added alone to the wells in the concentrations of 0.01 nM, 1 nM, 0.1 µM and 10 µM and incubated for 7 days. To mimic sequential or continuous combined HRT, the cells were either treated with 10^{-10} M estradiol for 3 days and a estradiol/progestin combination for further 4 days or with a estradiol/progestin combination for 7 days. After incubation for 7 days, cell proliferation was measured by ATP chemosensitivity test [5]. In brief, proliferation is quantified by measuring light emitted during the bioluminescence reaction of luciferine in the presence of ATP and luciferase.

ANOVA on the log values, which followed normal distribution, was used for statistical analysis. After that, Student's *t*-test of triplicates from the two different experiments was applied.

Results

The results for the effect of the progestins alone on cell proliferation are illustrated in Fig. **1** as the difference from the serum-stimulated control value percentage index of 100 %. For progesterone and levonorgestrel no significant changes were observed for progesterone and levonorgestrel over the entire concentration range tested. For chlormadinone acetate (CMA) the proliferation of MCF-7 cells was significantly stimulated at the concentrations of 0.1 and 10 µM. The same picture was observed for dienogest (DNG), gestodene (GSD), ketodesogestrel (KDG), medroxyprogesterone acetate (MPA), and norethisterone (NET). At the highest concentrations tested, 10 µM CMA, DNG, GSD, and KDG elicited a significantly higher effect on cell proliferation, nearly doubling cell numbers compared to MPA and NET.

Estradiol combined with the various progestins in a sequential manner with the result that estradiol at a concentration of 10^{-10} M alone elicited a 2 – 3-fold increase in cell proliferation (Fig. **2**). This increase was differently influenced by the progestins; P inhibited cell proliferation only at the highest concentration of 10 µM by about 23 %. CMA significantly reduced cell proliferation at all concentrations. The values ranged between 10 and 23 %.

DNG showed an increasing inhibitory effect with increasing concentrations between 4 and 15%. With GSD and KDG, cell proliferation was reduced at concentrations of 0.01 nM, 1 nM and 0.1 µM, whereas no inhibition was found at 10 µM. LNG and NET were not able to suppress estradiol-induced stimulation of cell proliferation. An inhibitory effect was observed for MPA only at 10 µM.

Continuous estradiol/progestin combination revealed the following results that are shown in Fig. **3**. A significant inhibition of cell proliferation was observed for all progestins. The values for P ranged from 20% to around 40%, CMA between 8 to 25%, DNG 5 to 12%, DSG between 18 and 32%, KDG 10 to 25%, LNG 5 to 23%, MPA 3 to 23% and NET between 5 and 8%.

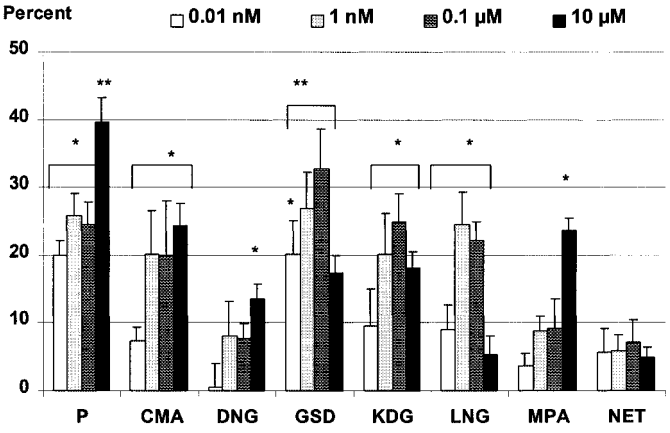

Fig. **3** Inhibition of estradiol (10^{-10} M) induced proliferation of MCF-7 cells by various progestins continuously combined with estadiol. P: progesterone, CMA: chlormadinone acetate, DNG: dienogest, GSD: gestodene, KDG: 3-ketodesogestrel, LNG: levonorgestrel, MPA: medroxyprogesterone acetate, NET: norethisterone (means ± SD, * $p < 0.05$, ** $p < 0.01$ vs. estradiol value).

Discussion

Before WHI, most epidemiological studies on HRT and the risk of breast cancer were applied mainly or solely on monoestrogen replacement therapy. Studies including combined estrogen/progestin treatment often fail to distinguish between the individual gestagens used. In those that do differentiate between different gestagens, such as the Magnusson study [6], the case numbers are too small for a statistically significant conclusion to be drawn on their effect on breast cancer. The WHI was the first study to cover large numbers of patients treated with a defined estrogen/progestin combination in a continuous regimen.

However, there is still a debate as to whether the type of regimen has any effect on the risk of breast cancer, and whether a continuous combined or a sequential combined HRT treatment should be favored. The Magnusson study showed both to carry a similar breast cancer risk in ever-users. Short-term use – less than 2 years – indicated a lower risk for continuous combined, while longer use of HRT – 10 years and more – indicated a lower risk for sequential combined HRT. Again, the conclusion is based on too few cases. The study of Ewertz [7] showed the continuous combined regimen to have a lower breast cancer risk compared to sequential combined, but was inferred from only 8 breast cancer cases studied.

Present epidemiological studies only show a slight increase in breast cancer [8] and seem to indicate a greater risk at developing more benign forms of breast cancer. However, breast cancer risk evaluation has to be taken very seriously since not many ways are known for reducing the breast cancer risk in the population.

Cell cultures tend to give different results depending on the exact experimental conditions. So, were curious as to whether cell cultures treated according to the continuous combined model would give different results from the sequential combined model, this being the first study to investigate this question comparing several progestins head to head. Our aim was to imitate the *in vivo* situation as closely as possible in order to gain a potential indication of the risk of breast cancer depending on the mode of treatment – continuous combined compared with sequential combined.

In our experiments, the C21-progestins, progesterone, chlormadinone acetate and medroxyprogesterone acetate showed different behaviors on cell proliferation. Progesterone, the natural progestin, had no effect on cell proliferation on its own. Sequentially combined with estradiol, progesterone inhibited cell proliferation only at the highest dosage of 10 µM to a slight extent. In continuous combination, progesterone showed an inhibitory effect at all concentrations being highest at 10 µM. Chlormadinone acetate seems to be similar effective in reduction of estradiol-stimulated proliferation in both regimens. MPA in combination with estradiol either sequentially or continuously combined only had a small inhibitory effect at the highest concentrations.

Differences in C19 progestin, dienogest, gestodene, 3-ketodesogestrel, levonorgestrel and norethisterone reactivity were also observed. Dienogest, a new C19 progestin with antiandrogenic properties, had an stimulatory effect at high concentrations on its own and a low reductive effect on estradiol-stimulated cell proliferation in both regimens. The "third generation" C19 progestins – gestodene and 3-ketodesogestrel – reacted similarly in inhibiting proliferation in both regimens, sequential and continuously combined with estradiol, at all concentrations tested. Surprisingly, levonorgestrel showed no reduction of estradiol-stimulated cell proliferation when combined in a sequential manner. In contrast, this progestin was able to inhibit proliferation when combined continuously. Our results from comparing sequential and continuous combined incubation with estradiol demonstrated that norethisterone had only a slight inhibitory effect on estradiol-stimulated proliferation, which was rather similar for all concentrations tested.

Previous cell culture studies on the effect of these progestins on the proliferation of MCF-7 cells have revealed the following:

Progesterone appears to have no significant effect at a concentration range of 10^{-7} M to 10^{-12} M on cell proliferation in the presence or absence of estradiol [9].

To our knowledge, the effect of CMA has not so far been investigated in MCF-7 cells. However, CMA was able to inhibit cell proliferation in the breast cancer cell line ZR-75-1, which expresses estrogen- and progesterone receptors [10].

In an animal experiment, dienogest has been shown to elicit potent anti-tumor activity against hormone-dependent cancer types [11].

3-ketodesogestrel and gestodene have already been shown to be able to inhibit cell proliferation of MCF-7 cells in the absence of estradiol. In the presence of estradiol, however, the inhibitory effect was restricted to a specific subclone [12].

Other research group found divergent results concerning the effect of levonorgestrel alone, that is, inhibiting and stimulating properties [12,13]. Levonorgestrel could only reduce estradiol-stimulated proliferation in specific subgroups of MCF-7 cells [12].

No effect was found on proliferation with MPA [12 – 14]. Only two studies examined the effect of MPA on estradiol-triggered MCF-7 cell stimulation, which is closer to the actual *in vivo* situation [12,15]. They both showed an inhibition of estradiol stimulated proliferation at the higher MPA concentrations tested.

So far, only a few *in vitro* studies have investigated the effect of NET on estradiol induced proliferation. Catherino et al. [16] showed NET to have a similar proliferative effect than MPA on the proliferation of MCF-7 cells compared to controls; unfortunately, they did not provide an estradiol control for comparison. Interestingly, they also studied NET together with estradiol in a further experiment using a smaller starting number of MCF-7 cells and included an estradiol control that resulted in NET having an antiproliferative effect on the estradiol-induced cells compared with estradiol control alone. In the study by Schoonen et al. [12], NET plus estradiol showed no significant inhibition on cell proliferation. The conflicting results of Schoonen and Catherino are probably the result of different culture conditions, such as differences in the media for seeding of the cells or their cultivation, or a different number of days of preculture before the test substances are added.

Some clinical studies have been conducted investigating the effect of ERT and HRT on the proliferation of normal breast epithelium in postmenopausal women. In a small clinical trial, progesterone was not found to increase proliferation of normal epithelial breast cells alone or in combination with estrogen over a period of 14 days [17]. Hargreaves et al. however neither found an increase of the proliferation rate with estrogen alone nor combined with various progestins, that is, norethisterone, levonorgestrel, tibolone and MPA, in postmenopausal women treated up to 5.5 years [18]. In contrast, a recent clinical study demonstrated that MPA in combination with conjugated equine estrogens increased epithelial proliferation in the normal postmenopausal breast in women treated up to 20 years [19]. The latter data are consistent with animal experiments investigating the mitogenic effect of estrogens + MPA [20,21]. Thus, the role of progestins on proliferation of normal breast epithelium remains unclear, but the type of progestin may be of importance.

To assess the possible efficacy of the various progestins in reducing breast cancer risk, it is useful to know their serum concentrations. The clinically relevant blood concentrations for the progestins most commonly used for HRT, MPA and NET range from 4×10^{-9} M to 10^{-8} M for MPA [22] and of around 10^{-8} M [23] for NET. According to our results, these progestins do not seem to be able to reduce breast cancer risk. For the other progestins – LNG, DNG, GSD, CMA, and KDG – similar concentrations have to be expected. Thus, all these progestins apart from DNG appear to elicit an antiproliferative effect to a slight extent, at least when combined continuously with estradiol.

Clinically, it also seems to be of interest that recent studies suggest that breast cancer risk may be influenced by the estradiol metabolism [24,25], whereby exogenic factors such as smoking can influence estradiol metabolism, and may thus further increase the risk [26]. In our own investigations, we found that estradiol metabolites act in a proliferative or antiproliferative manner on MCF-7 cells [27]. On the other hand, HRT influences estradiol metabolism according to administration mode [28] as well as progestin addition [29]. Further experiments on the influence of progestins on breast cancer risk should therefore also include estradiol metabolites.

In conclusion, our data indicate that the type of progestin may be important for influencing breast cancer risk when combined with estrogens in HRT. In addition, differential behaviors of progestins regarding sequentially or continuously combination with estradiol also seem to be important. According to our *in vitro* data, continuous combined therapy seems to be superior to the sequential regimen, independent of the type of progestin used. However, the results of cell culture experiments seem to vary considerably; a comparison of the various progestins should be done in the same experimental model. The inhibitory effect of most progestins on the estradiol-stimulated cell proliferation seems to be rather minimal. According to our results, the progestin used in the WHI study, MPA, might not be the best choice of progestin to reduce estradiol-induced breast cancer risk in long-term treatment. Therefore, further clinical studies are needed to serve in choosing the types of progestins, if any, with the lowest breast cancer risk.

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