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Comparison of the proliferative effects of estradiol and conjugated equine estrogens on human breast cancer cells and impact of continuous combined progestogen addition

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ABSTRACT

Objectives So far, most epidemiological studies investigating breast cancer risk and hormone replacement therapy have been conducted with conjugated equine estrogens (CEE). Recent trials indicate that the addition of progestogens may increase breast cancer risk. In the present study, we compared the effects of the human estrogen 17β-estradiol (E_2) with those of the main equine components of CEE, i.e. equilin (Eq) and 17α-dihydroequilin (Dheq) on the proliferation of human breast cancer cells. The proliferative effect of progestogen addition was also investigated.

Materials and methods The well-established human breast cancer cell line MCF-7 was used as an *in vitro* model. The proliferative effect of E_2, Eq and Dheq was tested in the concentration range 0.01–10 nmol/l. The progestogens progesterone, medroxyprogesterone acetate (MPA) and norethisterone (NET) were continuously combined with 0.1 nmol/l estrogen at concentrations of 0.01 nmol/l, 1 nmol/l, 0.1 μmol/l and 10 μmol/l. Proliferation was measured after 7 days by the adenosine triphosphate (ATP) chemosensitivity test.

Results All three estrogens increased the proliferation of MCF-7 cells by between 40 and 180%. The most proliferatively potent estrogen was E_2, followed by Eq and Dheq, which showed a slightly lower proliferative activity than E_2. The addition of progesterone inhibited E_2-induced proliferation by about 30%, but only at the high non-physiological concentration of 10 μmol/l. All three progestogens inhibited Eq-induced proliferation, although their effect tended to be low, with values between 5 and 40%. No progestogen reduced Dheq-induced proliferation by more than 20%. In contrast, MPA slightly increased the proliferation rate by about 5% at the high physiological concentration of 0.1 μmol/l when combined with Dheq. The same held true when MPA and NET were added at the high pharmacological concentration of 10 μmol/l, causing increases of about 10%.

Conclusions Our results indicate that equine estrogens have a proliferative action similar to that of 17β-estradiol. Continuous addition of progestogens does not result

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in any major reduction of proliferative potency. Some progestogens may even enhance the estrogen-induced proliferation of pre-existing breast cancer cells, particularly when combined with certain equine estrogens. However, in none of the tested circumstances do progestogens increase the proliferative effect of estradiol, and progesterone has no deleterious effect even at pharmacological levels, in contrast to progestogens.

INTRODUCTION

Increased breast cancer risk is a woman's most feared side-effect of hormone replacement therapy (HRT) in the postmenopause. Clinical trials indicate that the breast cancer risk increases by about 30% after more than 5 years of treatment[1,2]. Recent epidemiological studies suggest that progestogen addition may contribute to this increase; however, the patient numbers are too small to draw definitive conclusions[2–12]. More conclusive evidence can be expected after final assessment of the Women's Health Initiative (WHI) results become available from the still ongoing study arm receiving unopposed estrogen treatment. Furthermore, there are still open questions concerning type of estrogen–progestogen regimen, i.e. sequential versus continuously combined, which may differ in their risk profiles. Here, too, epidemiological trials have so far investigated only small subgroups[3,6–8,13–16], and contradictory results exist for different experimental models[17–20].

Most epidemiological studies have used conjugated equine estrogens (CEE) as the estrogenic component, while most experimental studies have tested estradiol. Until recently, the estrogens available for clinical use in the USA were almost exclusively CEE. This is in contrast to Europe, where preparations with estradiol have been on the market for about 20 years. Therefore, we decided to compare the natural estrogen 17β-estradiol with the main equine estrogenic components of CEE, equilin and 17α-dihydroequilin, with regard to their effects on the proliferation of human breast cancer cells. A secondary objective was to elucidate the effect that the addition of progesterone, medroxyprogesterone acetate (MPA) and norethisterone (NET) can have on the estrogen-stimulated proliferation of these cells. We focused mainly on continuously combined estrogen– progestogen, since we had already demonstrated in a recent study that continuously combined MPA or NET added to estradiol had a higher inhibitory potency, compared with sequential treatment, on estradiol-stimulated proliferation of the human breast cancer cell line MCF-7[19].

MATERIALS AND METHODS

Estradiol, progesterone, medroxyprogesterone acetate and norethisterone were purchased from Sigma Chemical (Munich, Germany). Equilin and 17α-dihydroequilin were purchased from Steraloids (Wilton, USA). The compounds were dissolved in ethanol and diluted by ethanol–phosphate-buffered saline (PBS) mixtures to yield a final ethanol concentration of < 1% per well.

MCF-7, a human estrogen and progesterone receptor-positive breast cancer cell line, was purchased from the European Collection of Cell Cultures, 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 with approximately 1000 cells per well in an assay-kit medium. Subsequently, the cells were incubated for 3 days with medium containing charcoal–dextran-treated serum. The estrogens were tested at concentrations of 0.01, 0.1, 1 and 10 nmol/l; to obtain a maximal proliferation rate the incubation time was set at 7 days. To mimic continuously combined HRT, the cells were treated with a combination of progestogen added to 0.1 nmol/l estrogen for 7 days. The progestogens were tested at concentrations of 0.01 nmol/l, 1 nmol/l, 0.1 µmol/l and 10 µmol/l. After incubation for 7 days, cell proliferation was measured by the adenosine triphosphate (ATP) chemosensitivity test, which has been established in our laboratory for several years[21].

Statistical analysis of the logarithm transformed values which followed normal distribution was first performed by analysis of variance (ANOVA) and then using Student's *t* test of triplicates from two different experiments.

RESULTS

Figure 1 presents results from the three estrogens tested on the proliferation of MCF-7 cells. Estradiol (E_2) showed the strongest stimulatory potency at the chosen concentration range of

0.01–10 nmol/l. Equilin (Eq) and 17α-dihydroequilin (Dheq) were slightly weaker than estradiol within the same range. E_2 increased proliferation by between 154 and 172%, compared with the control value ($p < 0.01$). The corresponding ranges for Eq and Dheq were between 40 and 130% and 81 and 125%, respectively ($p < 0.01$). The E_2-induced proliferation rate was constant over the entire concentration range, with Eq and Dheq exhibiting a moderate concentration–efficacy relationship.

Figure 2 depicts results for the continuous addition of progesterone, MPA and NET to a constant estrogen concentration of 0.1 nmol/l. At 0.01 nmol/l, all three progestogens had no effect on estradiol-stimulated proliferation and a minor effect on Dheq, and they inhibited equilin-stimulated proliferation by about 10% ($p < 0.05$).

At 1 nmol/l, there was still no effect on E_2-stimulated proliferation. Combined with equilin and 17α-dihydroequilin, all three progestogens

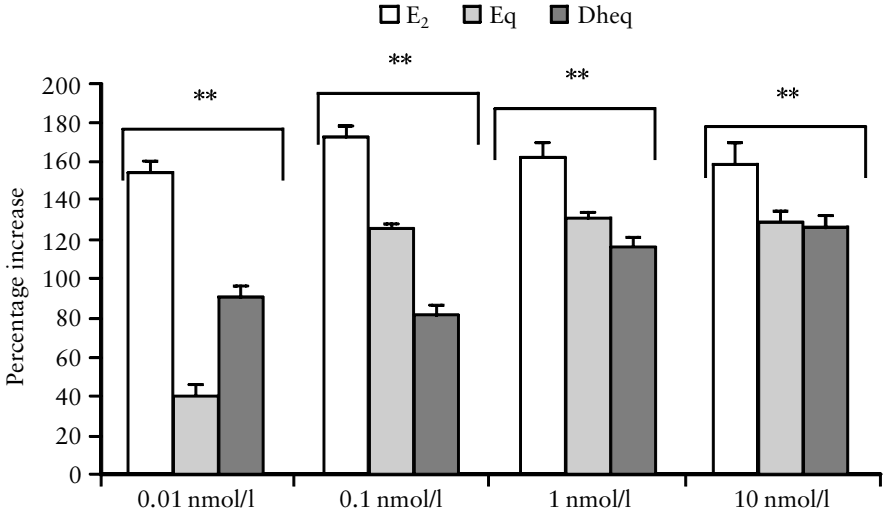

Figure 1 Percentage increase of proliferation of MCF-7 cells after addition of various estrogens. Values are shown as means ± SD. $^{**}p < 0.01$ vs. control value = 100%. E_2, 17β-estradiol; Eq, equilin; Dheq, 17α-dihydroequilin

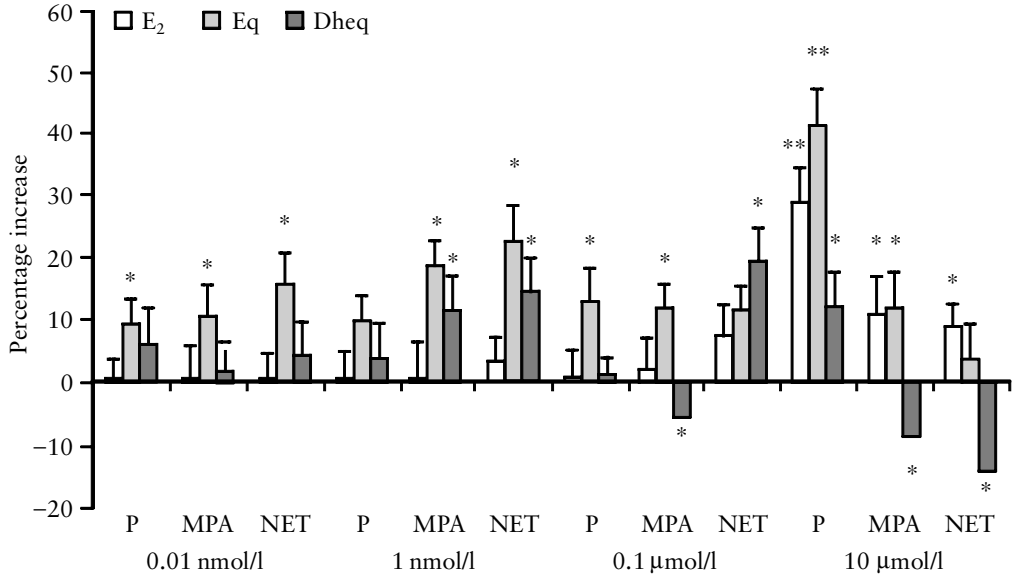

Figure 2 Changes of estrogen-stimulated growth of MCF-7 cells after addition of progestogens. Values are shown as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. estrogen alone value = 100%. E_2, 17β-estradiol; Eq, equilin; Dheq, 17α-dihydroequilin; P, progesterone; MPA, medroxyprogesterone acetate; NET, norethisterone

were able to inhibit proliferation, which was greatest for NET, by about 20% for Eq and about 12% for Dheq ($p < 0.05$).

At 0.1 μmol/l, all three progestogens still had a very slightly inhibitory effect on E_2-stimulated proliferation. When combined with Eq, a similar inhibition of about 10% was observed for all progestogens ($p < 0.05$). In the case of Dheq, progesterone had no effect, and NET inhibited proliferation by about 20% ($p < 0.05$). MPA showed a stimulatory effect of almost 5% ($p < 0.05$).

The story was different at a concentration of 10 μmol/l. Progesterone inhibited estrogen-stimulated growth by about 30% when combined with estradiol ($p < 0.01$), by 40% with equilin ($p < 0.01$) and by 10% with 17α-dihydroequilin ($p < 0.05$). When combined with E_2 and Eq, MPA reduced proliferation by about 10% ($p < 0.05$), while stimulating cell proliferation by about 10% when combined with Dheq ($p < 0.05$). A slight reduction was found when NET was combined with E_2 and Eq, a stimulation of about 15% being found in combination with Dheq ($p < 0.05$).

DISCUSSION

The MCF-7 line is a well-recognized *in vitro* cell model representing receptor-positive human breast cancer. This model is widely used to test the proliferative or antiproliferative efficacy of new substances, which may be considered effective therapeutic agents for human receptor-positive breast cancer[19,22].

The pharmacological dosages of conjugated equine estrogens (CEE) commonly used for HRT are 0.625 and 1.25 mg/day. These doses are comparable to 2 or 4 mg of natural estrogen, i.e. 17β-estradiol (E_2), given per day[23,24]. The clinically relevant blood levels achieved range between 50 and 200 pg/ml, equivalent to a concentration of 0.01–0.1 nmol/l. The clinically relevant blood concentrations of the three progestogens are in the region of 10 nmol/l progesterone when administered in a dosage of 200 mg/day[25], and in the range 4–10 nmol/l for MPA[26] and around 10 nmol/l for NET[27].

However, the assessment of blood level values may be limited, since it has been shown that tissue levels of estrogens are up to ten-fold higher than circulating concentrations. Moreover, it is well recognized that estrogens are synthesized in the breast tissue, and especially in tumor tissue, at concentrations that are remarkably higher than blood levels achieved under HRT[28].

Whereas dose–efficacy studies are easy to conduct with physiological estradiol, it is much more difficult to assess the efficacy of CEE. Equine estrogens are extracted from the urine of pregnant mares, not synthesized by the human body. CEE vary in composition, and recent investigations have shown that they can contain more than 200 steroidal components[29]. The major compounds are estrone, with a percentage ranging between 50 and 60%, equilin, between about 20 and 30%, 17α-dihydroequilin, between about 14 and 20%, 17α-estradiol, at about 3–10% and 17β-dihydroequilin, at about 0.5–4.0%[23,24]. Thus, we chose to test the first three estrogens, using estradiol instead of estrone, since the latter is rapidly converted intracellularly into the active 17β-estradiol.

The results of our study demonstrate that the proliferative effect of estradiol shows no clear-cut dose-dependency over the tested concentration range. As shown by our previous work, the proliferative action of estradiol declines at concentrations just above 10 nmol/l, and an anti-proliferative effect is observed at a concentration of 10 μmol/l[19]. In contrast, equilin and 17α-dihydroequilin exhibit a dose-dependent stimulation of cell proliferation with a similar potency. At least ten compounds are responsible for the activity of CEE, and over 200 steroids including progestogens and androgens can be found in CEE[23,24]. Thus, the true proliferative potency of CEE remains unclear, and the present results allow only a relative comparison of the main estrogenic components of CEE.

None the less, it is possible to assess the potency of the various progestogens to influence estrogen-induced proliferation. Our experiment demonstrated a distinct progestogen type- and concentration-dependency of this proliferative effect. At low concentrations, none of the progestogens was able to inhibit estrogen-stimulated proliferation in an unequivocal manner. At the high dosage of 10 μmol/l, only the natural progestogen, progesterone, inhibited cell proliferation when all three estrogens were added. Only minor inhibitory effects were found with MPA and NET when combined with E_2 and Eq. When combined with Dheq, stimulatory effects were observed, which started at lower concentrations of MPA than of NET. Our data suggest that these progestogens may have only a low risk-reducing potency when combined with estradiol or CEE in clinically relevant dosages, and may even increase cancer risk when present at high concentrations.

The actual intracellular concentrations of these progestogens are not known. Therefore, it cannot be excluded that higher concentrations of progestogens are present intracellularly and may have an impact on cell proliferation, especially when transformed breast cells are already present. Hence, low-dose administration of progestogens may be superior to high doses in long-term HRT treatment, since the effect of progestogen addition on the breast and the cardiovascular system still remains questionable. Therefore, the choice of progestogen in terms of type and dosage should be oriented towards their endometrial safety. On the other hand, high progestogen dosages are effective in treating breast cancer. However, the mechanism(s) of these very high-dose progestogen effects are currently unknown, and the doses may differ substantially from those used for HRT.

Few clinical studies have been conducted concerning the effects of estrogen replacement and HRT on the proliferation of normal breast epithelium in postmenopausal women. One small clinical trial demonstrated that progesterone given for 14 days decreased proliferation of normal epithelial breast cells, alone or in combination with estrogen[30]. In postmenopausal women treated for up to 5.5 years, Hargreaves and colleagues found no increase of the proliferation rate with estrogen, either alone or in combination with various progestogens, i.e. norethisterone, levonorgestrel, tibolone and MPA[31]. In contrast, one clinical study found that MPA in combination with conjugated equine estrogens increased epithelial proliferation in the normal postmenopausal breast of women treated for up to 20 years[32]. The latter data are consistent with animal experiments investigating the mitogenic effect of estrogens plus MPA[20,33]. The difference in the two clinical human studies may be partly attributable to the use of different proliferation markers. Although the role of progestogens in proliferation of normal breast epithelium still remains unclear, we are now aware that the progestogen type may also be of importance.

Our *in vitro* experiments were limited by the fact that the results of cell models strongly depend on culture conditions. Therefore, the results are not directly comparable from laboratory to laboratory. *In vitro* results can only hint at mechanisms, and are only conditionally transferable to the *in vivo* state. Furthermore, the proliferation of breast cancer cells depends on multiple factors, with growth factors produced by surrounding stromal cells being discussed as primary influences[34,35]. In a recent experiment, we found that estradiol seems to be the primary proliferating factor for MCF-7 cells when directly compared with various growth factors; however, combination of estradiol with some growth factors such as epidermal, fibroblast and insulin-like growth factors leads to an enhanced proliferation rate (manuscript in preparation).

It can be concluded that the estrogenic effects found in our *in vitro* experiments and the modifications caused by progestogen addition may significantly influence the growth of pre-existing malignant human breast cells. However, in none of the tested circumstances do progestogens increase the proliferative effect of estradiol, and progesterone has no deleterious effect even at pharmacological levels, in contrast to progestogens.

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