Comparison of the effects of 17α-ethinylestradiol and 17β-estradiol on the proliferation of human breast cancer cells and human umbilical vein endothelial cells

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Summary

Objective: Little is known about the direct comparison between 17α-ethinylestradiol (EE2) and 17β-estradiol (E2) on pre-existing breast cancer cells and on their angiogenetic effects. In this study we investigated the effect of both estrogens on the proliferation of MCF-7 cells, a human breast cancer cell model, and on human umbilical vascular endothelial cells (HUVECs).

Methods: The steroids were tested in the concentration range of 10^{-10} to 10^{-5} M. The proliferation of MCF-7 and HUVEC cells was measured after five and six days by the crystal violet staining technique.

Results: In the concentration range from 10^{-10} to 10^{-5} M both estrogens showed a proliferative effect in the MCF-7 cells, E2 having a gradually declining effect on proliferation with increasing concentration while EE2 showed a constant proliferative effect over a large concentration range. At the highest concentration tested E2 had no effect on proliferation while EE2 even inhibited growth. In the HUVEC cells both estrogens showed a slightly significant stimulatory effect at the lowest concentration, and no significant effect at the remaining concentrations. EE2 again inhibited cell growth at the highest concentration.

Conclusions: At the serum concentrations seen in hormone replacement therapy, EE2 appears to have less proliferative effect on breast cancer cells compared with E2, while both estrogens appear to have similar effects on endothelial cells.

Key words: 17α-Ethinylestradiol; 17β-Estradiol; MCF-7; HUVEC; Proliferation.

Introduction

17α-ethinylestradiol (EE2) is clinically of immense importance being the estrogen component in oral contraceptives (OCs), based on its higher bioavailability and higher local concentration in the endometrial cells compared with 17β-estradiol (E2) and therefore its ability to maintain cycle stability during treatment of women in reproductive phase at a lower dose than E2. EE2 also plays a role in hormone replacement therapy (HRT), due to endogenous aromatisation of a small percentage of norethisterone acetate to EE2 [1, 2]. Aromatisation of norethisterone is probably of little clinical significance at 1 mg norethisterone in combination with 2 mg estradiol taken orally, even though the EE2· serum concentration can constitute a tenth of the resulting E2 serum concentration. It could however be of significance at a dose of 5 and 10 mg norethisterone which might lead to EE2 serum concentrations similar to ones seen for 30 μg EE2 taken orally. In addition EE2 plays a role in HRT since it has recently been reintroduced to the HRT market. E2, mainly in the esterified forms such as E2 valerate or E2 benzoate, on the other hand is used in HRT, since it has fewer side-effects than EE2.

Obtaining a clearer understanding of the effect that EE2 and E2 have on the risk of breast cancer and on the growth of cancer cells with respect to angiogenesis is of great interest. Initiation of breast cancer occurs many years before presentation of the disease which makes it important to elucidate effects on pre-existing breast cancer cells. E2 on its own can promote the growth of estrogen receptor positive breast cancer. Many epidemiological studies on HRT do not clearly distinguish between the different types of estrogens and are often mainly based on conjugated equine estrogens. The "Collaborative Group on Hormonal Factors in Breast Cancer" which reanalysed over 90% of the epidemiological studies worldwide came to the conclusion that there is a small increase in the risk of breast cancer for HRT users, which increases with duration of use [3]. After cessation of use the increase diminishes slowly over the following five years. Analysing the use of OCs the same group found a small increase in risk during the use of OCs, with a diminishing risk for the next ten years after cessation of use [4].

Angiogenesis is concerned with the formation of new blood vessels. Angiogenetic properties are not only of importance in healthy processes such as wound healing but also in the field of cancer such as tumour development and distant metastasis, since the tumour cells require a good blood supply for growth [5,6]. Vascular endothelium has been shown to have functioning estrogen binding sites and estrogen receptor-associated proteins [7]. All stages of angiogenesis such as stimulation of cell attachment, migration and proliferation of endothelial cells were induced by estradiol stimulation [8].

Two well established human cell culture models were used, MCF-7 cells and human umbilical vein endothelial cells (HUVECs). MCF-7 cells, derived from a pleural effusion of a woman with metastasing breast carcinoma,

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are estrogen receptor positive epithelial cells. This common human breast cancer model has been used in antitumour and pharmacological studies including mechanistic investigations on estrogens, anti-estrogens and progestins [9]. HUVEC cells are derived from the umbilical cord after childbirth and retain their differentiated characteristics for several passages. They have been used to gain a better insight into vascular pharmacology, angiogenesis and atherosclerosis. Surprisingly the literature research revealed that no direct comparison between these two estrogens has been carried out on these well established cell culture models. Since OCs and HRT products are often taken over many years it is important to investigate the effects of these two estrogens.

Materials and Methods

17α-ethinylestradiol and 17β-estradiol were purchased from Sigma Chemical Co., Munich, Germany and dissolved in ethanol and diluted to 10% ethanol with phosphate buffered saline (PBS). The final steroid concentrations in the wells were 10^{-10} to 10^{-5} M, with the final ethanol concentration in the wells being 0.1%. Dulbecco's modified Eagle's medium (DMEM), phenol-free DMEM and MCDB 131 medium were obtained from Gibco BRL, Eggenstein, Germany and fetal calf serum (FCS) from Seromed Biochrom KG, Berlin, Germany. The heparin was purchased from Sigma Chemical Co., Munich, Germany and the bovine endothelial cell growth factor (ECGF) from Boehringer Mannheim, Mannheim, Germany.

MCF-7 cells were acquired from DSMZ, Braunschweig, Germany. Prior to the experiment, the MCF-7 cells were maintained in 5% FCS in DMEM supplemented with 0.3 mg/ml glutamine, 5 ng/ml bovine insulin and 100 U/ml penicillin plus 100 µg/ml streptomycin. The cells were seeded and incubated for 24 h in the above medium using 20% FCS. The cells were then washed with PBS, followed by incubation in 5% dextran-coated charcoal-treated FCS (to remove any steroids) in phenol red free DMEM using the same supplements as described above for the maintenance medium. The HUVEC cells were acquired from PromoCell, Heidelberg, Germany. The HUVEC cells were maintained, seeded and cultured throughout the experiment in 10% FCS in MCDB 131 medium supplemented with 0.3 mg/ml glutamine, 100 U/ml penicillin plus 100 µg/ml streptomycin, 20 µg/ml ECGF and 50 µg/ml heparin.

MCF-7 cells were seeded at 500 cells per well into 96 well plates in 20% FCS-DMEM medium. After 24 h, the cells were washed with PBS and replaced with 5% stripped FCS phenol-red free DMEM medium, and preincubated for three days prior to treatment, to increase sensitivity of the cells to estradiol. The cells were then treated with either E2 or EE2 in the concentration range from 10^{-10} to 10^{-5} M for five days. Ethanol controls were performed containing the same final ethanol concentration as the test substances i.e. 0.1% ethanol. The HUVEC cells were seeded at 2,000 cells per well into 96 well plates in 10% FCS-MCDB 131 medium. The cells were pre-incubated for two days and then treated with either E2 or EE2 in the same concentration range as above for six days. Again ethanol controls were performed containing a final concentration of 0.1% ethanol in the wells.

The determination of proliferation of the MCF-7 and the HUVEC cells was based on the crystal violet staining technique of Kueng *et al.* [10] which relies on the staining of the cell nuclei. In brief, the cells were fixed with 11% glutaraldehyde, followed by washing of the cells with distilled water, and staining with a 0.1% crystal violet solution. The cells were then rewashed with distilled water, solubilised with a 10% acetic acid solution and shaken, prior to the reading of the plates at 600 nm using an enzyme-linked immunosorbent assay (ELISA) reader. Statistical analysis of the results was carried out using ANOVA and the Student's t-test (n = 12).

Results

As seen in Figure 1 for the MCF-7 cells, and as expected, E2 significantly stimulated the proliferation of the cells by 38.7% ± 5.3 to 67.4% ± 14.7, in the concentration range from 10^{-10} to 10^{-7} M. No significant stimulatory effect was seen at 10^{-6} and 10^{-5} M E2. EE2 also showed a significant stimulating effect on the cells but in a slightly wider range of concentrations, including 10^{-6} M, by

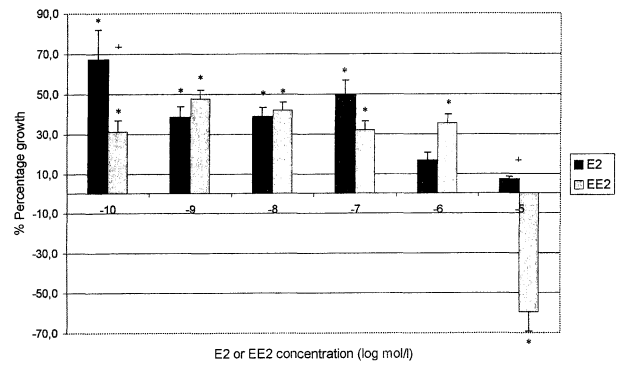

Figure 1. — Effect of 17ß-estradiol (E2) and 17α-ethinylestradiol (EE2) on the proliferation of MCF-7 cells. The cells were preincubated for three days and then incubated with either E2 or EE2 for five days in the concentration range from 10^{-10} to 10^{-5} mol/l. Controls were treated with the equivalent ethanol concentration (final ethanol concentration of 0.1%).
* = significant difference between control and estrogen induced growth at p = 0.05 level.
+ = significant difference between the two estrogens at p = 0.05 level.

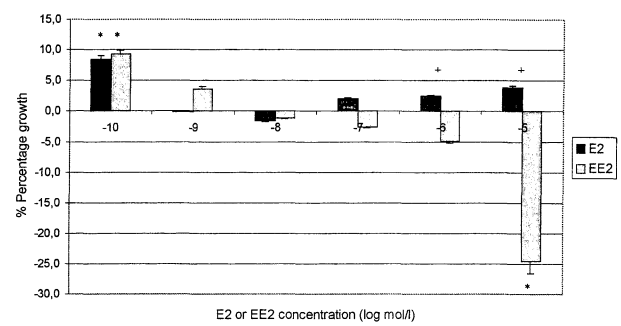

Figure 2. — Effect of 17ß-estradiol (E2) and 17α-ethinylestradiol (EE2) on the proliferation of HUVEC cells. The cells were preincubated for two days and incubated for six days with either E2 or EE2 in the concentration range from 10^{-10} to 10^{-5} mol/l. Controls were treated with the equivalent ethanol concentration used in the test substances (final ethanol concentration of 0.1%).
* = significant difference between control and estrogen induced growth at p = 0.05 level.
+ = significant difference between the two estrogens at p = 0.05 level.

31.0% ± 5.9 to 47.6% ± 4.6. At 10^{-5} M an abrupt reversal of the effect on the MCF-7 cells was seen for EE2 with a very strong significant inhibitory effect on the growth of the cells of -59.6% ± 9.5. It is interesting that our results seem to indicate a gradual trend in decline of proliferation for E2 from 10^{-10} to 10^{-5} M in comparison with a relatively constant effect on proliferation of the cells from 10^{-10} M to 10^{-6} M seen for EE2, with a sudden inversion from stimulation to inhibition between 10^{-6} and 10^{-5} M. Significant differences between the two estrogen treatments were found for the highest and the lowest concentration tested, i.e. 10^{-10} M and 10^{-5} M. E2 had about twofold the stimulating effect at 10^{-10} M (67.4% ± 14.7 vs 31.0% ± 5.9) and only half the proliferative effect at 10^{-6} M (16.7% ± 4.1 vs 35.3% ± 4.7) compared with EE2.

In the HUVEC cells, as seen in Figure 2, both E2 and EE2 showed a slightly significant proliferative effect on the cells at the lowest concentration tested, i.e. 10^{-10} M of 8.5% ± 0.4 and 9.3% ± 0.6, respectively. At the concentrations from 10^{-9} to 10^{-6} M both E2 and EE2 showed no significant effect on the growth of the cells compared with the controls. At 10^{-5} M a sudden change from no effect to a comparably strong significant inhibition of growth of the HUVEC cells was observed for EE2 of -24.5% ± 2.1. For this model no significant effect was observed for E2 at 10^{-5} M.

Discussion

Our results show that both E2 and EE2 stimulate the growth of MCF-7 cells in a similar fashion, yet leading to an obvious and abrupt change for EE2 from stimulating the proliferation of the cells to inhibiting the growth of the MCF-7 cells and a gradual change for E2 from a strong proliferative effect to no effect on the cells. Thus E2 has its strongest proliferative effect at 10^{-10} M, being about twice the effect that EE2 has on the MCF-7 cells at the same concentration, the differences in effect between the two estrogens being significant. While increasing concentrations of E2 results in a gradually declining effect on stimulation from a fairly strong to low effect, EE2 appears to produce a fairly constant and medium stimulative effect on proliferation over a wide range, losing its proliferating effect between 10^{-6} and 10^{-5} M and leading to strong inhibition of growth. It is conceivable that EE2 might involve an earlier inversion compared with E2 for which an inversion from a proliferating to an inhibiting effect on the MCF-7 cells might have become apparent if we had included higher concentrations in our experiment, which was not feasible. For cell culture studies it is important to compare the effect of inversion only in the same model under the same culture conditions used.

The phenomena of transformation from proliferation to loss of proliferating effect and/ or inhibition, depending on the dose, has been known for E2 and for EE2 and has been used clinically in the past with the use of high doses of E2 or EE2 in the treatment of breast cancer patients. Due to the fairly strong side-effects of estrogens this form of treatment has been replaced by other treatments such as antiestrogens.

In the HUVEC cells we observed a similar yet much weaker trend for the estrogens on the growth of cells. At the lowest concentration tested both estrogens had a significant proliferative effect on the cells. Higher concentrations of both E2 and EE2 had no significant effect on the cells, yet again an inversion of effect was seen for EE2 which showed a relatively strong inhibitory effect on the HUVEC cells between 10^{-6} and 10^{-5} M, while E2 did not yet show any inhibitory effect at the highest concentration tested. A previous study of ours [11] showed a gradual decline in proliferative effect of E2 which then turned into an inhibitory effect of the growth of HUVEC cells at 10^{-5} M. Since HUVEC cells are derived from a pool of human umbilical vein cells, variations between pools and differing culture conditions have to be taken into account, thus substances for comparison are best used alongside in the same study.

The literature research did not reveal any comparative studies on the proliferative effect of E2 and EE2 in MCF-7 or in HUVEC cells over a concentration range. A recent study comparing various MCF-7 sublines did show a great variability in proliferative response to estrogens, suggesting the adherence to one subline for comparative studies [12]. No direct comparisons were found for animal studies regarding breast cancer or angiogenesis of the two estrogens.

A study comparing the metabolism of the two estrogens in MCF-7 cells showed E2 to undergo phase I metabolism to estrone with the equilibrium in favour of E2 formation and phase II metabolism to estrogen sulphates, while EE2 showed no evidence of phase I metabolism and conversion to the 3-sulphate conjugate [13]. In a previous study of ours we found that E2 exhibits its inhibitory action in the HUVEC cells via certain metabolites at high doses such as 2-methoxyestradiol, 2-hydroxyestrone, 2-hydroxyestradiol, 4-hydroxyestrone and 4-hydroxyestradiol while having a slight stimulatory effect at lower concentrations [11]. In the case of breast cancer cells it has been known for a long time that high doses of E2 are useful in the treatment of breast cancer albeit connected with many side-effects. The same effect was clinically found for EE2 in high doses which was in the past used in the treatment of breast cancer. Clinically relevant concentrations found in women taking E2 in HRT would be in the order of 3.3×10^{-10} M [14] and in the order of 4.6×10^{-10} M for OC use with EE2 [15]. Recently EE2 has been reintroduced in the United States for HRT at a dose of 5μg for the treatment of climacteric symptoms and osteoporosis prophylaxis.

Cell culture studies are a means of observing trends and mechanistic effects which are not easily obtained otherwise. Since epidemiological studies are difficult as too many factors are involved, it is important to achieve an understanding of the effect of individual components on certain organs. Cell culture studies require careful interpretation since culture conditions play an important role in determining the results. A cell culture model cannot reproduce the complex clinical situation but can

reflect many characteristics of the original tissue such as enzyme and receptor types so that one can focus on individual factors possibly involved in the *in vivo* situation. A model can attempt to approximate the clinical situation and help in the elucidation of possible mechanisms involved but never replace prospective clinical or epidemiological studies.

Since both estrogens elicit a proliferative action on MCF-7 cells, this might be an indication of an increased risk of breast cancer. Since EE2 does show a weaker effect than E2 at the lowest concentration tested, which is closest to the serum concentration expected for the low dose taken for HRT, it appears to have a lower stimulating activity on estrogen receptor positive breast cancer cells compared with E2. EE2 is taken in a much lower dose than E2, but is then metabolised at a slower rate than E2. The two Lancet studies [3, 4] which investigated the risk of breast cancer for HRT and for OC, did show a higher relative risk for women who had used HRT for five years or longer compared with the relative risk of current OC users. Yet it is difficult to interpret these studies since one of the main risk factors for breast cancer is age, thus a direct epidemiological comparison between the risks of E2 and EE2 is hardly possible. In addition the study on HRT was mainly based on women using conjugated estrogens. Regarding endothelial cells both estrogens seem to have a similar yet weak stimulatory action at the lowest concentration tested, which is of importance concerning the blood supply of tumours. This would suggest a slight stimulatory action for both estrogens on the production of new vessels. Gaining an even better understanding of 17α-ethinylestradiol is important since it is a standard component of oral contraceptives and due to its recent reintroduction to hormone replacement therapy (HRT) as well as it being the metabolic by-product of norethisterone.

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