ABSTRACT

Most oral contraceptives (OC) contain a progestin in combination with an estrogen, and the progestin component in OC includes one of the following 19-nortestosterone derivatives: norethynodrel; norethindrone; or norgestrel (levonorgestrel). It is well known that estrogens promote the growth of breast cancer. However, progestins have recently also been implicated in the development of breast cancer. We have compared and contrasted the ability of synthetic progestins to stimulate the proliferation of cultured human breast cancer cells and examined their possible mechanism of action.

We found that some progestins used in OC were able to stimulate the growth of estrogen receptor-positive (ER⁺) MCF-7 and T47D/A18 human breast cancer cells but not ER⁻ MDA-MB-231, BT-20, and T47D/DA18 breast cancer cells. However, two other progestins, MPA and R5020, which are not used in OC, were either not able to stimulate or only slightly stimulated growth. The potency of norethynodrel [median effective dose (EC₅₀) = 4 x 10⁻⁸ M] and norethindrone (EC₅₀ = 3 x 10⁻⁸ M) was greater than norgestrel (EC₅₀ = 2 x 10⁻⁷ M) in MCF-7 cells. EC₅₀ (EC₅₀ = 8 x 10⁻¹³ M) was an even more potent stimulator of growth. More importantly, the progestin-induced growth stimulation was blocked by the antiestrogens 4-hydroxytamoxifen andICI 164,384 but not the antiprogestin RU486.

This study provides direct evidence that the 19-nortestosterone derivatives in OC have estrogenic properties and suggests that activation of ER, but not progesterone receptor, is the growth-stimulatory mechanism for these synthetic progestins. Our results may help to explain the conflicting evidence linking OC and breast cancer risk. A rigorous evaluation of the "total" estrogenic potential of OC might produce a better correlation with breast cancer risk.

INTRODUCTION

OC³ have been used by women to control ovulation, and most OC contain an estrogen in combination with a progestin (1). There is a concern that OC may increase the risk of breast cancer because most of the known risk factors for breast cancer are related to steroid hormones (2, 3). However, there is conflicting evidence regarding the increased risk of breast cancer in women taking OC (4–12). Also, the inconsistency of laboratory evidence has made the interpretation of the relationship between the use of OC to breast cancer risk more difficult (13–16).

It is well known that estrogens promote the growth of breast cancer (17). However, progestins have also been implicated in the development of breast cancer (4, 5, 18, 19), but the underlying mechanism is not known. Most of the progestin components of OC include the 19-nortestosterone derivatives norethynodrel, norethindrone, and norgestrel (levonorgestrel) (1, 20–22) (Fig. 1), all of which have been shown to bind to PR with high affinity (23–25) and have potent prostegestational activity. However, we have recently demonstrated that one of the progestin components used in OC, norethindrone, can stimulate breast cancer cell growth and decrease transforming growth factor β2 and β3 mRNA levels (26), actions generally ascribed to estrogen action. It is therefore important to determine whether the progestin components of OC in general can initiate cell replication.

The action of steroid hormones is mediated by the steroid's cognate receptors located in the nucleus and the hormone response elements in the target genes. Antiestrogens (17, 27–29) and antiprogestin (30–32) are proving to be powerful tools in the understanding of gene regulation mediated by steroid hormones because of their ability to specifically block the actions of the relevant steroid hormones.

In this study, we investigated whether the commonly used progestins in OC could stimulate the growth of human breast cancer cells and determined whether these progestins could act through the ER. We found that not only norethindrone but also the other 19-nortestosterone derivatives, norethynodrel and norgestrel, can stimulate the growth of ER⁺ but not ER⁻ human breast cancer cells. More importantly, we demonstrated that these progestins induced the activity of a CAT reporter gene containing an ERE, and this induction could be blocked by the addition of antioestrogens but not antiprogestin.

MATERIALS AND METHODS

Cell Culture. The MCF-7 cell line (33) was originally obtained from Dr. Dean Edwards (Department of Medicine, Health Sciences Center at San Antonio, San Antonio, TX), who obtained the stock from the Michigan Cancer Foundation. The MCF-7 cells were between passages 470 and 510. MCF-7 cells were grown in minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum (heat inactivated), 0.29 mg/ml 1-glutamine, 100 units/ml penicillin plus 100 µg/ml streptomycin, 6 mg/ml bovine insulin (Sigma Chemical Co., St. Louis, MO), 0.35 g NaHCO₃/liter, and 25 mM N-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

The T47D/A18 and T47DC4 cells (34) were cloned from T47D cells originally obtained at passage 81 from the American Type Culture Collection (Rockville, MD). The T47D/A18 and T47DC4 cells were between passages 200 and 220. T47D/A18 cells were cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (heat inactivated), 0.29 mg/ml 1-glutamine, 100 units/ml penicillin plus 100 µg/ml streptomycin, 6 mg/ml bovine insulin, and 0.35 g NaHCO₃/liter.
T47DC4 cells were cultured in dextran-coated charcoal-stripped fetal bovine serum and phenol red-free RPMI 1640. For MDA-MB-231 cells, the culture condition was the same as previously described for MCF-7 cells except for the replacement of 5% calf serum with 10% fetal bovine serum (heat inactivated). MDA-MB-231 cells were between passages 30 and 40.

Medium components were obtained from Gibco (Grand Island, NY) unless otherwise stated. Cells were routinely passed by an initial wash with calcium- and magnesium-free Hanks' balanced salt solution followed by a 5-min incubation with 1 mM EDTA, also in Hanks' solution. Cell stocks were kept in 162-cm² flasks (Costar Corp., Cambridge, MA) in a humidified atmosphere of 95% air/5% CO₂ at 37°C. The cells were negative for the presence of Mycoplasma.

Hormone Treatment. E₂, norethynodrel, norgestrel, norethindrone, and MPA were purchased from Sigma Chemical Co. R5020 was purchased from New England Nuclear Products (Boston, MA). 4-OHT and ICI 164,384 were obtained from ICI Pharmaceuticals (Macclesfield, England). RU486 was a gift from Roussel Uclaf (Romainville, France). All compounds were prepared in a concentrated form in 100% ethanol and diluted 1:1000 (v/v) into cell culture medium. Final ethanol concentrations in the media were 0.1 to 0.2%.

Growth-Response Assay. Cells (except T47DC4) were plated in media containing phenol red and whole serum at a density of 14,000 cells/well into 24-well plates (Costar Corp.) in triplicate. The next day, media were changed to phenol red-free media containing dextran-coated charcoal-stripped serum, and media were changed again the next day. Cells were deprived of estrogen for a total of 4 days before the addition of compounds. Media that contained compounds were then added for 6 days, and cells were collected by scraping. Cytosols were obtained from Abbott Laboratories (N. Chicago, IL).

RESULTS

Growth Stimulation of ER⁺ Human Breast Cancer Cells by E₂, Norethynodrel, Norgestrel, and Norethindrone and Blockade by Antiestrogens but not Antiprogestin. Previously, we showed that norethindrone, a 19-nortestosterone derivative which is a commonly used progestin in OC, stimulated the growth of MCF-7 cells (26). We have now investigated whether other 19-nortestosterone derivatives, which are also used in OC, can stimulate human breast cancer cells to grow. We chose norethynodrel and norgestrel (levonorgestrel) because these two progestins are also widely included in OC (39). We found that, at a concentration of 10⁻⁶ M, not only norethindrone (5.6-fold over control) but also norethynodrel (7.2-fold over control) and norgestrel (4.8-fold over control) could stimulate the growth of MCF-7 cells in culture (Fig. 2A). Fig. 2A is representative of data from 7 independent experiments.
potency of norethynodrel ($EC_{50} = 4 \times 10^{-8} \text{ M}$) or norethindrone ($EC_{50} = 3 \times 10^{-8} \text{ M}$) was greater than norgestrel ($EC_{50} = 2 \times 10^{-7} \text{ M}$). However, $E_2$ ($EC_{50} = 8 \times 10^{-13} \text{ M}$) was even more potent as a growth stimulator than the progestins. $E_2$ at a concentration of $10^{-10} \text{ M}$ stimulated cell growth (9.4-fold over control). R5020, a progestin lacking the 19-C group like the 19-nortestosterone derivatives, stimulated minimal cell growth at $10^{-6} \text{ M}$ as reported by other investigators (40). The progestin MPA, which is not a 19-nortestosterone derivative, did not stimulate the growth of MCF-7 cells. Similar results were obtained using other ER$^+$ human breast cancer T47DA18 (Fig. 2B) and ZR-75-1 cells (25).

Antiestrogens (17, 27-29) block $E_2$-induced growth in MCF-7 cells. The addition of the antiestrogens 4-OHT (Fig. 2, C and D) or ICI 164,384 (data not shown) also blocked the growth induced by the three progestins. Competitive reversal of the blockade by the antiestrogens was observed. Interestingly, the antiestrogen RU486 did not influence the growth stimulation induced by these progestins at a concentration of $10^{-7} \text{ M}$ (Fig. 2D) or $10^{-6} \text{ M}$ (data not shown). Furthermore, it is important to note that the addition of progestins did not completely block the $E_2$-induced cell proliferation to the control level (Fig. 3).

The ability of the 19-nortestosterone derivatives and MPA to regulate the growth of ER$^+$ human breast cancer MDA-MB-231, BT-20, and T47DC4 cells was also examined. Neither these progestins nor $E_2$ altered the growth of ER$^+$ MDA-MB-231 (Fig. 4), BT-20, or T47DC4 (data not shown) human breast cancer cells.

Induction of pvtERE-TK-CAT Activities by $E_2$ and Progestin Components in OC and Blockade by Antiestrogens but not Antiprogestin. Since the antiestrogens 4-OHT and ICI 164,384 blocked the growth stimulation induced by norethyno-

drel, norgestrel, and norethindrone, we believed that the growth stimulation induced by these progestins was mediated by ER.

To test this hypothesis, the pvtERE-TK-CAT (ERE-CAT) (37) was used as the reporter gene to examine whether these progestins were estrogenic in inducing the expression of the CAT gene downstream from an ERE. ERE-CAT consists of an ERE derived from the vitellogenin 2A gene and the thymidine kinase promoter derived from herpes simplex virus linked to the gene for CAT. We observed that norethynodrel induced the ERE-CAT activity in a concentration-dependent fashion.
ESTROGENICITY OF PROGESTINS

When the concentration of E₂ reached \(10^{-11}\) M, a slight induction of ERE-CAT activity was observed, and this induction reached a maximum level at a concentration of \(10^{-8}\) M (7.4-fold over control). Antiestrogens 4-OHT and ICI 164,384 blocked both norethynodrel- and E₂-induced ERE-CAT activities (Fig. 5, A and B). However, when the concentrations of norethynodrel and E₂ were increased to \(3 \times 10^{-6}\) and \(10^{-7}\) M respectively, norethynodrel and E₂ reversed the blockade by antiestrogens on ERE-CAT activity.

Progestins that stimulated the growth of human breast cancer cells were able to induce the activity of ERE-CAT (Fig. 6). Fig. 6 is representative of data from 5 independent experiments. The extent of induction of ERE-CAT activity by these progestins (\(10^{-6}\) M) was identical to that observed for E₂ (\(10^{-10}\) M) (Fig. 6). We found that antiestrogens 4-OHT and ICI 164,384 could block the progestin-induced ERE-CAT activities (Figs. 6 and 7). Furthermore, the antiprogestin RU486, which blocks progestin binding to the PR and forms nonactive complexes with the hormone response element (31, 32), did not block the progestin-induced growth stimulation (Fig. 2D) and did not prevent the expression of ERE-CAT (Fig. 7). Therefore, these progestins behave more like estrogens with respect to cellular proliferation.

In contrast to the ER⁺ MCF-7 cells, in which norethynodrel, norgestrel, norethindrone, and E₂ induced the expression of ERE-CAT, in ER⁻ MDA-MB-231, BT-20, and T47DC4 cells we observed no induction of ERE-CAT activity by any of these compounds (Fig. 8).

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**Fig. 4.** Effects of various progestins and E₂ on the growth of ER⁻ MDA-MB-231 cells. Cells were seeded at a density of 14,000 cells/well into 24-well plates in triplicate, deprived of estrogens for 4 days, and then treated with compounds for 5 days. Media were changed every 2 days. □, control (0.1% ethanol); ○, E₂; ■, norethynodrel; △, norethindrone; △, norgestrel; ▲, R5020; ●, MPA.

(EC₅₀ = \(3.2 \times 10^{-9}\) M) (Fig. 5A). Fig. 5A is representative of data from 5 independent experiments. Norethynodrel at concentrations of \(10^{-13}\) to \(10^{-11}\) M induced no activity. When the concentration reached \(10^{-9}\) M some induction was observed, which demonstrated a maximum stimulation at \(10^{-6}\) M (7.4-fold over control). E₂ also regulated the ERE-CAT activity in a concentration-dependent fashion (EC₅₀ = \(3.2 \times 10^{-11}\) M) (Fig. 5B). When the concentration of E₂ reached \(10^{-11}\) M, a slight induction of ERE-CAT activity was observed, and this induction reached a maximum level at a concentration of \(10^{-8}\) M (7.4-fold over control). Antiestrogens 4-OHT and ICI 164,384 blocked both norethynodrel- and E₂-induced ERE-CAT activities (Fig. 5, A and B). However, when the concentrations of norethynodrel and E₂ were increased to \(3 \times 10^{-6}\) and \(10^{-7}\) M respectively, norethynodrel and E₂ reversed the blockade by antiestrogens on ERE-CAT activity.

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**Fig. 5.** Dose response of norethynodrel (A) and E₂ (B) on the induction of proviRTK-CAT activity in the presence and absence of antiestrogens 4-OHT and ICI 164,384 in MCF-7 cells. Cells were plated into 10-cm-diameter dishes at a density of \(8 \times 10^5\) cells in phenol red-free medium containing stripped calf serum. Cells were then deprived of estrogen for 6–7 days before transfection, and media were changed every other day. Transfections were done by the calcium phosphate method with \(10 \mu\)g of proviRTK-CAT plasmid together with \(5 \mu\)g of pCMV/β plasmid/plate. After transfection, cells were treated with compounds for an additional 48 h, and β-galactosidase activity and CAT activity were then determined. CAT reactions were performed with cell extracts containing equal amounts of β-galactosidase activity.
Results were observed in an additional 4 independent experiments. Norethynodrel (10^{-6} M), norgestrel (10^{-6} M), norethindrone (10^{-6} M), R5020 (10^{-6} M), MPA (10^{-6} M), and E2 (10^{-10} M) were used. Similar results were observed in an additional 4 independent experiments.

Effects of E2 and Progestins on the TK-CAT (pBL.CAT.2), pvitERE15-TK-CAT, pvitERE15 mt-TK-CAT, and pGRE15T-TK-CAT (pGRE15T) Activities in MCF-7 Cells. In order to confirm that norethynodrel, norgestrel, and norethindrone could bind to ER and induce ERE-CAT activity, we also used a mutant ERE construct (pvitERE15 mt-TK-CAT) (Fig. 9A), which cannot be induced by the ER-ligand complex (37). We found that these progestins could not induce the CAT activities of parental plasmid pBL.CAT.2 and the pvitERE15 mt-TK-CAT containing the mutant ERE (Fig. 9B).

Klock et al. (37) have shown that ERE and PRE/GRE are closely related but distinct. We showed that these progestins also induced the CAT activity of pGRE15T (Fig. 9A), and the addition of RU486 blocked this progestin-induced CAT activity (Fig. 9C).

Regulation of PR Level by Progestins. To determine whether the progestins that were able to stimulate cellular proliferation and induce ERE-CAT activity could regulate the synthesis of an endogenous estrogen target gene, the change in PR level was examined after treatment of MCF-7 cells with the progestins and E2. The progestins that were estrogenic (i.e., stimulate cellular proliferation and induce ERE-CAT activity) were also able to induce endogenous PR synthesis (Table 1). Therefore, these progestins can regulate gene expression in a manner similar to that of E2. The progestins MPA or R5020, which were not distinctly estrogenic, did not induce PR synthesis. Interestingly, the antiestrogen 4-OHT was able to block the norethindrone-induced PR synthesis. This agrees with our hypothesis that the progestins in OC are estrogenic and that antiestrogens are able to block their actions.

Discussion

We have investigated the effects of progestins on the proliferation of human breast cancer cells and determined the possible mechanism underlying their action. We conclude that the 19-nortestosterone derivatives norethynodrel, norgestrel, and norethindrone are estrogenic in stimulating the growth of ER+ human breast cancer cells and that this action is mediated through ER.

Plasma progestin concentrations in women taking OC is in the range of 10^{-7} to 10^{-8} M (39, 41, 42), consistent with the levels we used in vitro. Therefore, our finding that the growth of mammary epithelia in culture can be stimulated by the progestins in OC supports the hypothesis that these compounds might do the same in vivo (16). In addition, our study provides further support for the possibility that women taking OC for an extended period of time will maintain this risk of developing breast cancer. Our results (Fig. 3) demonstrate that a modest protective effect at best could be expected from these progestins in the prevention of breast cancer. Unlike antiestrogens, the progestins do not have a profound effect on estrogen-stimulated growth. The dominant action of 19-nortestosterone derivatives is as a growth promoter. This position is supported by the epidemiological data, which show that women are not protected from breast cancer by OC use.

Recently, Battersby et al. (43) demonstrated that the ER was decreased and PR was increased by the progestins in OC in the normal breast. In addition, Williams et al. (44) showed that OC use increased cell proliferation in normal breast and suggested that OC use is a risk factor for breast cancer. This is consistent with the observation we obtained in cultured human breast cancer cells. The ability of the progestin components in OC to stimulate the growth of human breast cancer cells and to induce PR synthesis in a manner that was blocked by antiestrogens but not antiprogestin led us to speculate that these progestins were estrogenic in stimulating the growth of human breast cancer cells and that this action was mediated through ER. Furthermore, it is possible that 19-nortestosterone derivatives could act
Fig. 9. Effects of various progestins and E2 on the induction of pBL.CAT.2, pvitERE15-TK-CAT, pvitERE15 mt-TK-CAT, and pGRE15T-TK-CAT activities. A, plasmid constructs of various reporter genes. B, effects of various progestins and E2 on the induction of pBL.CAT.2, pvitERE15-TK-CAT, and pvitERE15 mt-TK-CAT activities. Cells were seeded, transfected, and assayed for CAT activity as described in Fig. 5. The concentrations of compounds used were: E2, 10^{-10} M; norethynodrel, 10^{-6} M; norgestrel, 10^{-6} M; norethindrone, 10^{-6} M; R5020, 10^{-6} M; and MPA, 10^{-6} M. C, effects of norethynodrel on the induction of pGRE15T-TK-CAT activity. MCF-7 cells were seeded, and 10^{-10} M E2 was added for 4 days before the transfection. Cells were then transfected, and norethynodrel at various concentrations was added for an additional 48 h. CAT was assayed as described in "Materials and Methods.”

through ER (45), since it was suggested that the estrogenic action of androgens is mediated through ER in human breast cancer MCF-7 cells (46). Several reports have described the estrogenic actions of 19-nortestosterone derivatives (25, 46-48). However, they did not have sufficient evidence to demonstrate that ER, but not PR, is involved. Our approach using hormone response element-CAT constructs proves the mechanism is via ER.

It is well known that steroid regulation of gene transcription in target cells is mediated by the steroid’s cognate specific high-affinity intracellular receptor proteins (49-51). These receptors act as transcription factors that can regulate gene expression positively or negatively by interacting with specific DNA sequences (hormone response elements) (49-51). Estrogen regulation of target gene transcription is mediated by estrogen receptors, which interact with the ERE sequence located in target genes (52, 53). Meyer et al. (54) showed that ER cannot activate transcription controlled by a PRE/GRE in vivo or bind to a PRE/GRE in vitro, nor can PR bind to a synthetic palindromic ERE. Therefore, only ER, and not PR, can bind to a synthetic palindromic ERE. We favor the hypothesis that 19-nortestosterone derivatives that are progestins bind directly to ER and then interact with the ERE in the target gene because (a) only ER and not PR interacts with the vitERE (54) used in our study; (b) these progestins induce the activities of only ERE-CAT but not the mutant ERE-CAT nor the parental plasmid; (c) other progestins do not induce ERE-CAT activities; (d) we do not observe any induction of the ERE-CAT activity in ER- cells; (e) antiestrogens but not antiprogestin block the proges- tin-induced cell proliferation and ERE-CAT activities; and (f)
antiprogestin is able to block the progestin-induced GRE-CAT activity but not the progestin-induced growth and ERE-CAT activity.

We also ruled out the possibility that these progestins were converted into aromatized estrogens for their actions, since the aromatase inhibitors 4-hydroxyandrostenedione and aminoglutethimide did not block the growth stimulation induced by these progestins (data not shown). Nevertheless, it is still possible that these progestins were converted into estrogenic metabolites, as suggested by other investigators (55–58). This does not invalidate the conclusion, however, that the 19-nortestosterone derivatives are indeed estrogenic. Further studies will be necessary to confirm this possibility. In addition, we were able to show that the action of norethindrone was not due to the presence of an estrogenic contaminant such as mestranol. When the high-performance liquid chromatography-purified norethindrone peak was collected and used for both growth and ERECAT assays, we observed the same results as reported here (data not shown). Therefore, this study suggests that 19-nortestosterone derivatives in OC can activate ER to stimulate the growth of ER+ human breast cancer cells and provides further support for the dual effects of the 19-nortestosterone derivatives used in OC.

The observation that R5020 was able to induce ERE-CAT activity only at high concentrations is nevertheless intriguing. R5020 may exhibit some weak estrogenic activity at high concentrations. This is consistent with the observation by Hissom and Moore (40) that R5020 stimulated proliferation of T47D human breast cancer cells. It is important to note that R5020 is also a steroid lacking the 19-C group like 19-nortestosterone derivatives. Therefore, it is reasonable to believe that R5020 may have weak estrogenic actions at high concentration.

Our results may help to explain the conflicting evidence linking OC and breast cancer risk. A rigorous evaluation of the "total" estrogenicity of OC on breast cancer cells might produce a better correlation with breast cancer risk. This study provides direct evidence that 19-nortestosterone derivatives have intrinsic estrogenic properties. In addition, these results should also prove to be helpful in evaluating the long-term safety of OC and in understanding the underlying mechanism of the proposed toxicities. Since estrogen is implicated in the development of breast cancer, we believe that less estrogenic progestins should be selected for inclusion in OC. There is a decrease in ovarian and endometrial carcinoma in OC users but no decrease in breast cancer incidence. Alteration in the formulation could in the future produce a decrease in breast cancer risk by reducing the "total" estrogenicity of the product.

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Estrogenic Potential of Progestins in Oral Contraceptives to Stimulate Human Breast Cancer Cell Proliferation

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