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EFFECTS OF PROGESTINS ON THE PROLIFERATION OF ESTROGEN-DEPENDENT HUMAN BREAST CANCER CELLS UNDER GROWTH FACTOR-DEFINED CONDITIONS

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Summary—The effect was studied of four different synthetic progestins (Org 30659, gestodene, 3-ketodesogestrel and levonorgestrel) on the proliferation of the 17β estradiol (E2)-dependent human breast cancer cell line MCF7. All progestins were found to stimulate proliferation, but only at high pharmacological dosages. Moreover, like estrogens the progestins at high concentrations synergistically stimulated MCF7 cell proliferation together with low concentrations of insulin. This stimulatory effect could be blocked by antiestrogens, but not by antiglucocorticoids and antiprogestins. This suggests that growth stimulation by these progestins (or their metabolites) occurs through crossreaction with the E2 receptor (ER). This is confirmed by the observation that the strong synthetic progestin Org 2058 does not stimulate proliferation. The absence of a progesterone receptor (PR)-mediated growth response seems not to be due to aberrant PR expression in these cells; 27,000 receptors (K_d 1.7×10^{-10} M) per cell were present under growth-assay conditions.

Growth stimulation by E2 in the absence or presence of insulin, is slightly inhibited or unaffected by the progestins, respectively. Our data do not support a role for the recently identified gestodene binding sites [Colletta *et al.*, *J. Steroid Biochem.* **33** (1989) 1055–1061] in mediating gestodene effects on breast cancer cells: gestodene and 3-ketodesogestrel, a compound that does not bind to these gestodene binding sites, showed a similar biological activity. The effects of the progestins on the MCF7 breast cancer cell line, indicate that the use of these compounds at very high concentrations may be unfavourable, but do not support a role for them in directly stimulating breast tumor proliferation at the low progestin concentration which are reached in the serum in oral contraceptive users.

INTRODUCTION

The effect of oral contraceptives on breast tumor development remains a controversial issue [1]. In contrast to the situation in endometrial cancer, in which a protective effect has been claimed, long-term use has been associated in some studies with an increased risk of breast cancer. However, conclusive proof for this is missing, and it is not known if breast cancer risk is different for the various formulations of oral contraceptives which are presently available.

Oral contraceptives contain both estrogens and progestins. Since a part of the human breast tumors contain receptors for these steroids, a direct regulation of tumor cell proliferation seems important in the possible effects on tumor development. Although the estrogenic compounds are rather invariant, various synthetic progestins at different dosages are used by manufacturers, which can lead to different side-effects of the contraceptives. Differences have been reported in the effect of various progestins on *in vitro* growth of breast tumor cells [2–4].

Recently, a binding site for the synthetic progestin gestodene has been described to which other progestins did not bind [5]. Moreover, data were presented recently that gestodene inhibits breast tumor cell proliferation *in vitro* [4]. This in itself is not surprising since other progestins have also been found to inhibit *in vitro* proliferation of breast tumor cells [2, 3, 6–9]. Especially, estrogen (E2)-induced proliferation is impaired [6, 9]. Controversially, stimulation of proliferation by progestins of the breast tumor cells has also been reported in the absence of E2 [3, 7, 8]. Since stimulation of the receptors of epidermal

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growth factor (EGF) and insulin has been reported, the response to progestins may be modulated by the additional presence of these factors [7, 10, 11]. This stresses the importance of studying progestin effects on such cells under defined conditions.

So far no detailed comparison between the effects of various progestins on breast tumor cells has been made under growth factor- and steroid-defined culture conditions. We have recently described a culture system in which the regulation of proliferation of breast tumor cell lines by steroids and polypeptide growth factors can be studied under defined conditions. We used media supplemented with serum in which growth factors were chemically inactivated and steroids removed [12]. As a result of this mitogen depletion the E2-dependent human breast cancer cell line MCF7 becomes quiescent in the G1/G0 phase of the cell cycle, while it can be stimulated to enter the cell cycle with the synergistic combination of low concentrations of insulin and E2 [12]. This quiescent state is not observed in conventional serum that is treated only to remove endogenous steroids, since this still contains growth factors causing background mitogenic activity. Thus, under our culture conditions MCF7 cells display their strict insulin- and E2-dependence that has been established *in vivo* (in nude mice), under physiological conditions [13].

Under these defined conditions we have compared the effect of four different progestins, among which gestodene, on the proliferation of the E2-dependent human breast cancer cell line MCF7. We have studied the effects on these cells in combination with physiological concentrations of various mitogens that are important in growth regulation of hormone-dependent breast cancer cells (reviewed in [14]), and which thus may be expected to modulate the response to progestins.

EXPERIMENTAL

Materials

A phenol red-free 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DF) was obtained from Gibco (Grand Island, NY). Trypsin and the EDTA used for cell culture were obtained from Flow Laboratories (Irvine, Scotland). Fetal calf serum (FCS) was purchased from Integro (Linz, Austria), bovine insulin, E2, human transferrin and bovine serum albumin (BSA) were from Sigma (St Louis, MO). The progestins Org 30659 (17-hydroxy-11-methylene-19-nor-17α-pregna-4,15-dien-20-yn-3-one), gestodene (13-ethyl-17-hydroxy-18,19-dinor-17α-pregna-4,15-dien-20-yn-3-one), 3-ketodesogestrel (13-ethyl-17-hydroxy-11-methylene-18,19-dinor-17α-pregn-4-en-20-yn-3-one), levonorgestrel (13-ethyl-17-hydroxy-18,19-dinor-17α–pregn-4-en-20-yn-3-one), Org 2058 (16α-ethyl-21-hydroxy-19-nor-pregn-4-en-3,20-dione), medroxyprogesterone acetate (MPA; 17α-acetoxy-6-methylpregn-4-en-3,20-dione) and the antiprogestin Org 31710 (6β, 11β, 17β)-11-[4-(dimethylamino)phenyl]-6-methyl-4′,5′-dihydro[estra-4,9-diene-17,2′(3′H)-furan]-3-one were provided by Organon International (Oss, The Netherlands). RU486 was provided by Roussel-Uclaf (Romainville, France) and 4′-hydroxytamoxifen by ICI Pharmaceuticals (Macclesfield, England). All steroids and antihormones were dissolved in 96% (v/v) ethanol, and were stored as concentrated stock solutions at −20°C. The final ethanol concentration in cultures did not exceed 0.2%. [^{3}H]Org 2058 (53 Ci/mmol) was from Amersham (Amersham, England). [methyl-^{3}H]Thymidine (77.9 Ci/mmol) was purchased from NEN (Boston, MA). DCC-FCS was prepared by treating FCS with dextran coated charcoal (DCC) to remove steroids, as described [12]. DCC-SH-FCS was prepared by a treatment with dithiothreitol to inactivate polypeptide growth factors [15], followed by a DCC-treatment, as described previously [12].

Cell culture

MCF7 cells were kindly provided by Dr C. Quirin-Stricker (Institut de Chimie Biologique, Faculté de Médicine, Strasbourg, France), and were cultured on phenol red-containing DF medium supplemented with 5% FCS and buffered with bicarbonate. The cells were passaged twice a week using trypsin and EDTA, and they were grown in a humidified atmosphere containing 7.5% CO_2. For experiments only exponentially growing, 4-day-old cultures were used. The cells were free of *Mycoplasma* contamination.

Cell proliferation

The effect of mitogens on MCF7 cells on DNA synthesis was tested in the absence of phenol red [16], essentially as described previously [12]. In short, cells were plated at a density of 1.0×10^4/cm^2 in DF containing 30 nM selenite, 10μg/ml transferrin and 0.2%

BSA (referred to as DF^+), supplemented with 5% DCC-FCS. After 24 h this medium is changed to DF^+, in which the cells become quiescent [12]. After another 24 h this medium is replaced by DF^+ supplemented with 10% DCC-SH-FCS. Subsequently the compounds to be tested were added in 2-[bis(2-hydroxyethyl)amino] ethane-sulfonic acid (50 mM; pH 6.8) buffered DF^+. After an additional 4 days of incubation the total DNA content per well was assessed by fluorescent staining with Hoechst 33258.

Analysis of variance was used to test for differences in the means of the data points [17]. Differences were considered significant when P values were 0.05 or less.

DNA synthesis

The cells were treated similarly as the cells in which proliferation was assessed (see above), except that 2.0×10^4 cells were plated/cm^2. 20 h after mitogen addition [^{3}H]thymidine (1.0μCi/ml) was added, and after another 8 h incubation the cells were washed with trichloroacetic acid (TCA) and then lysed with NaOH, followed by liquid scintillation counting, as described [18]. Statistical analysis was carried out as described above.

Progesterone receptor (PR) assay

A whole cell PR assay was used. Cells (2.0×10^4/cm^2) were plated in 6 well tissue culture plates and treated as described. Subsequently, the cells were washed twice with HEPES (20 mM; pH 7.2) buffered DF, supplemented with 0.2% BSA. Then the cells were incubated for 1 h at 37°C in the same medium, containing increasing amounts of labeled Org 2058 (3×10^{-11}–3×10^{-9} M) in the absence and presence of a 200-fold excess of unlabeled Org 2058. At the end of the assay the cells were washed 3 times with the above medium. Then the cells were lysed in 0.1 N NaOH, and radioactivity was counted. The data were analyzed according to the method of Scatchard [19].

Transfection and CAT assays

MCF7 cells, suspended in 5 ml DF^+ supplemented with 5% DCC-FCS, were plated at a density of 2×10^4 cells/cm^2 in a 6 cm diameter tissue culture dish. After 24 h the cells were incubated for 5 h with calcium phosphate-precipitated plasmid DNAs. The reporter plasmid used to determine PR-dependent transcriptional activity (pG29G-tkCAT; [20]) was generously provided by Dr Muller. Directly after transfection the cells were washed three times with DF^+ and fresh medium containing DCC-FCS (5%) and the hormones were added. The cells were incubated for another 24 h after the beginning of the hormonal treatment. The cells were then harvested and CAT activity was measured as described previously [21].

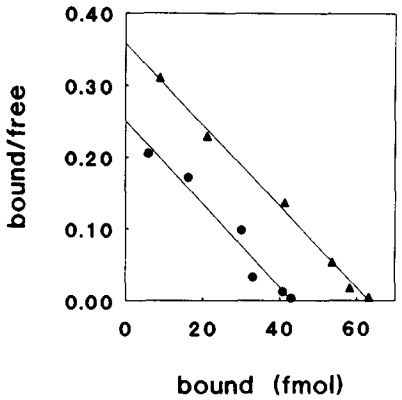

Fig. 1. Binding of [^{3}H]Org 2058 to MCF7 cells. MCF7 cells were treated similarly as in the cell growth experiments: cells were grown for 24 h in DF^+ supplemented with 5% DCC-FCS, followed by a 24 h incubation period in DF^+ only (●). Control cells were grown for 2 days in 10% FCS supplemented DF, and were at the same cell density at the beginning of the binding experiment (▲). Scatchard plot of specific binding (binding in the presence of 200-fold excess unlabeled subtracted from total binding), expressed per 10^6 cells.

RESULTS

Long-term retention of E2 by breast tumor cells has been reported [22], and for this reason depletion protocols of several weeks in E2-free medium are often used before initiation of an experiment. Since, however, expression of the PR in breast tumor cells is E2-dependent [23, 24], long-term steroid-depletion leads to a loss of PRs [25]. Therefore we used an equally effective 48 h procedure to deplete the cells from mitogens [12]. The results in Fig. 1 show that this protocol prevented to a large extent PR downregulation in MCF7 cells. Control cells, cultured in FCS supplemented medium, expressed 40,000 receptors per cell ($K_d = 1.8 \times 10^{-10}$ M), while at the end of our depletion protocol 27,000 receptors per cell ($K_d = 1.7 \times 10^{-10}$ M) were present.

The effect of the different progestins on the proliferation of MCF7 cells is shown in Fig. 2. In the absence of other steroids and growth factors, the progestins hardly influenced proliferation [Fig. 2(a)]. However, in combination

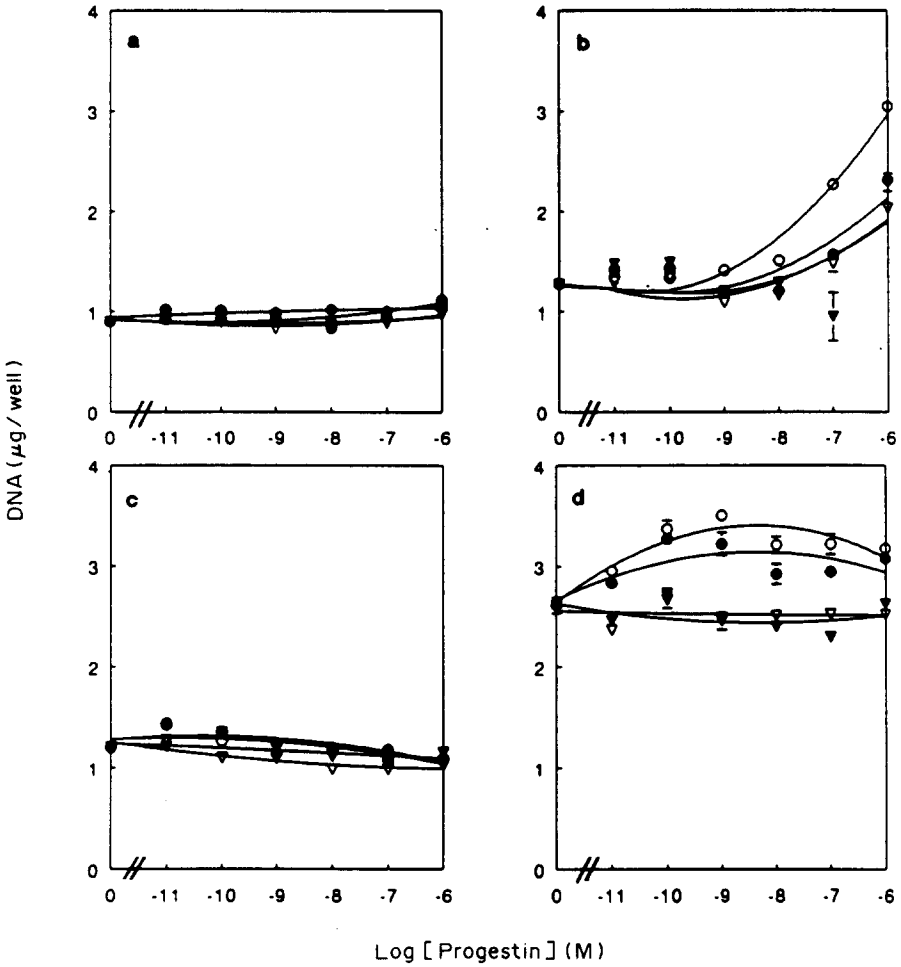

Fig. 2. The effect of progestins on the proliferation of MCF7 cells. Org 30659 (○), gestodene (●), 3-ketodesogestrel (▽) and levonorgestrel (▼) were added to quiescent MCF7 cells, in combination with the following additives: (a) vehicle only; (b) insulin at 10 ng/ml; (c) 10^{-9} M E2; and (d) the combination of insulin at 10 ng/ml and 10^{-9} M E2. After 4 days the total amount of DNA/well was determined as a measure for proliferation. Each point represents the mean of three determinations ± SEM. This experiment was repeated with similar results. No error bar is drawn when it is smaller than the marker.

with insulin at 10 ng/ml [Fig. 2(b)] or 25 ng/ml (not shown) all compounds synergistically stimulated proliferation at 10^{-6} M. Although Org 30659 was more stimulatory than the other compounds in the experiment shown in Fig. 2(b), the combined data of three independent experiments did not show any significant difference in this respect between the four compounds at 10^{-6} M (not shown).

We have shown previously that E2 alone is a weak mitogen for MCF7 cells, but in combination with low concentrations of insulin or insulin-like growth factors (IGFs) E2 synergistically stimulates proliferation of these cells [12, 25]. The weak mitogenic effect of E2 alone was inhibited slightly by all the progestins tested [Fig. 2(c)]. However, proliferation induced by the synergistic combination of E2 with insulin at suboptimal concentrations was not inhibited by the progestins [Fig. 2(d)]. In fact, under these circumstances Org 30659 and gestodene appeared to be stimulatory at physiological concentrations. However, this effect was not seen in the combined data of three experiments (at 10^{-9} M; not shown).

The effects on proliferation as shown in Fig. 2 closely resembled the effects on the induction of DNA synthesis 24 h after mitogen addition (Fig. 3). This rapid effect on DNA synthesis suggests that the progestins, like estrogens [18, 21], directly influence proliferation of MCF7 cells. The similarity of the dose–response curves for stimulation of MCF7 DNA synthesis after 24 h (Fig. 3) and cell proliferation after 4 days (Fig. 2) with progestins suggests that the high concentrations of progestins necessary to

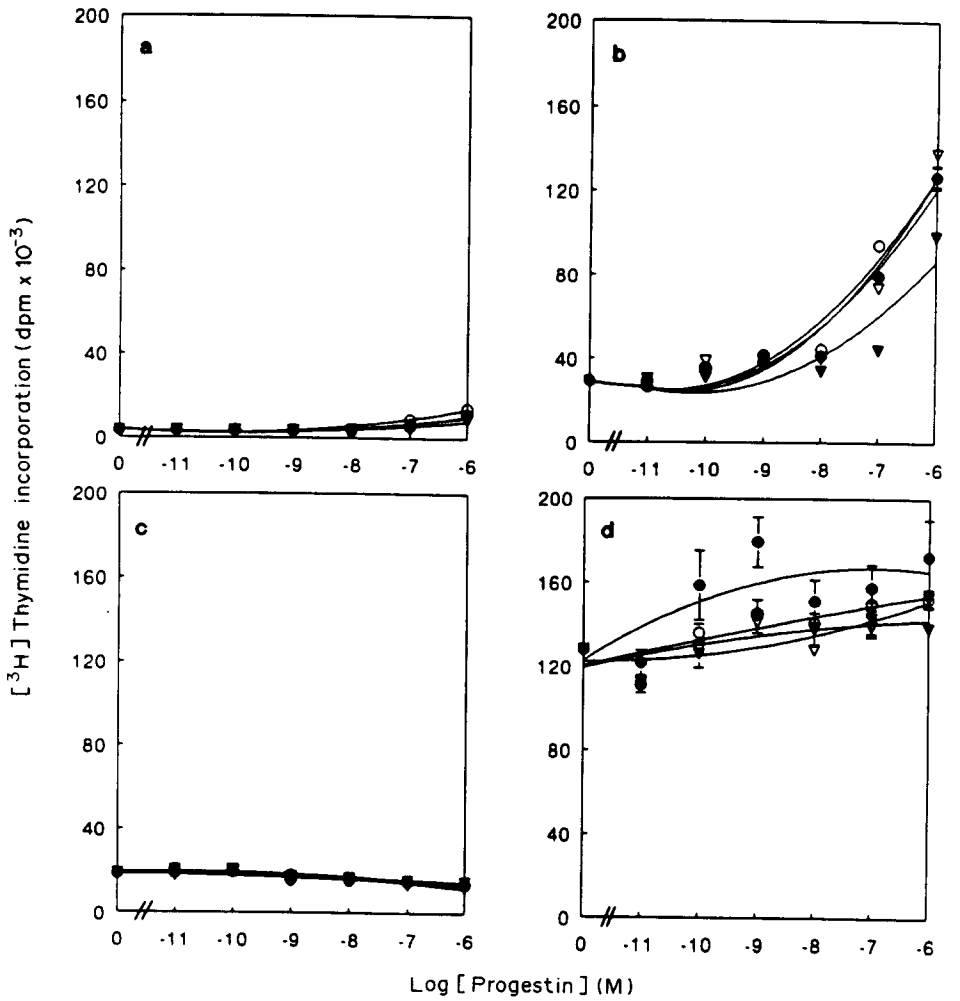

Fig. 3. The effect of progestins on DNA synthesis of MCF7 cells. Org 30659 (○), gestodene (●), 3-ketodesogestrel (▽) and levonorgestrel (▼) were added to quiescent MCF7 cells, in combination with the following additives: (a) vehicle only; (b) insulin at 10 ng/ml; (c) 10^{-9} M E2; and (d) the combination of insulin at 10 ng/ml and 10^{-9} M E2. After 24 h [^{3}H]thymidine incorporation/well was determined as a measure for the amount of DNA being produced. Each point represents the mean of three determinations ± SEM. This experiment was repeated with similar results. No error bar is drawn when it is smaller than the marker.

stimulate proliferation during the 4 day period of the growth experiments are not caused by degradation of the progestins. We hypothesized that this requirement for pharmacological concentrations of progestins reflected a low affinity binding of these progestins (or their metabolites) to another steroid receptor. The most likely candidate seemed the E2 receptor (ER) since estrogens can stimulate MCF7 growth. Moreover, a similar synergistic effect with insulin has been found for either E2 or high concentrations of the progestins (see above).

The results in Fig. 4 show that the stimulatory effect of high concentrations of these progestins (or their metabolites) is indeed likely to be due to crossreactivity with the ER. The stimulation could be abolished by the antiestrogen hydroxytamoxifen at a concentration (10^{-7} M) that blocks the E2 response of the cells (Fig. 4). At this concentration this antiestrogen specifically inhibited the E2 response, since the growth inhibition could be reversed by adding 10^{-7} M E2 (data not shown). Moreover, no inhibition of insulin (10 ng/ml)-induced proliferation was observed at this concentration of hydroxytamoxifen.

Figure 4 also shows that neither the antiglucocorticoid and antiprogestin RU486 nor the antiprogestin Org 31710 showed any specific inhibition of the progestin-induced effect. Combined with the observation that other progestins (the strong progestin Org 2058, and MPA) hardly stimulated the MCF7 cells, even at

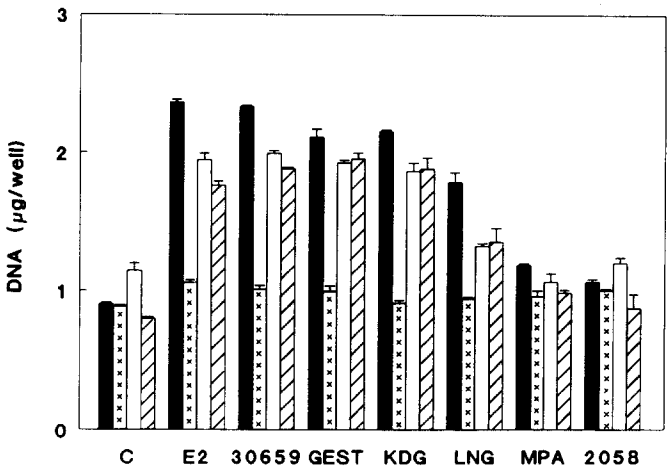

Fig. 4. The effect of antihormones on MCF7 proliferation induced by synthetic progestins at pharmacological concentrations. The mitogens were added, together with 10 ng/ml of insulin, at the following concentrations: E2, 10^{-9} M; Org 30659, gestodene (GEST), 3-ketodesogestrel (KDG), levonorgestrel (LNG), MPA, and Org 2058 at 10^{-6} M. The mitogens were tested either alone (■), or in the additional presence of 4′-hydroxytamoxifen; (10^{-7} M; ⊠), RU 486 (10^{-7} M; □) or Org 31710 (10^{-6} M; ▨). After 4 days the total amount of DNA/well was determined. This is a representative of three separate experiments. Each point represents the mean of three determinations ± SEM.

10^{-6} M, this indicated that the high affinity PR present in MCF7 cells (see above) seems not to be important in stimulation of proliferation under these conditions.

The lack of PR-mediated growth response seems not to be due to a defect in the PR in our MCF7 cells, leading to a loss of hormone-dependent transcriptional activity: while a transiently transfected PRE-CAT construct was silent in the absence of hormone, it was dose–dependent and clearly activated by Org 2058 (data not shown).

It has been reported that the number of EGF receptors in MCF7 cells can be stimulated by progestins [10]. In the MCF7 cell line, which contains low numbers of EGF receptors ([10, 26] van der Burg *et al.*, unpublished), and is hardly stimulated by EGF [12, 26, 27], such an upregulation of receptors could be physiologically important and stimulate the response to external EGF [or autocrine transforming growth factor-α (TGFα)]. However, as can be seen in Fig. 5, the response to EGF is not significantly enhanced by progestins.

DISCUSSION

We have found that under growth factor-defined conditions various progestins slightly inhibit E2-induced proliferation, depending on

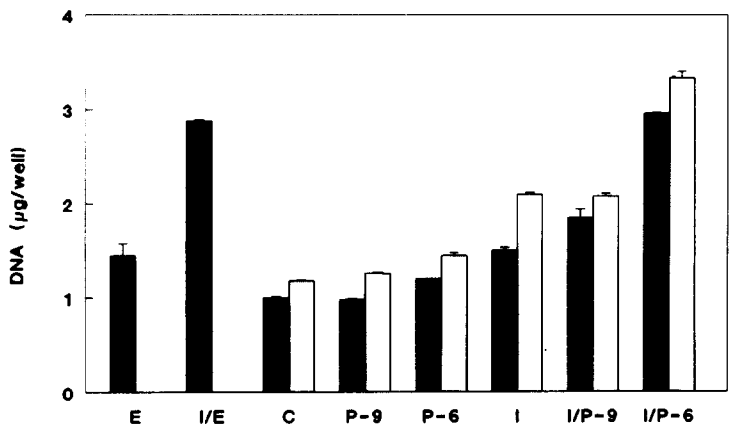

Fig. 5. The effect of a synthetic progestin on EGF-induced proliferation of MCF7 cells. The mitogens were added at the following concentration: E2, 10^{-9} M (E); insulin (I), 10 ng/ml; vehicle only (C); Org 30659 (P), 10^{-9} M (−9) or 10^{-6} M (−6). All incubations were in the absence (■) or presence (□) of EGF (10 ng/ml). After 4 days the total amount of DNA/well was determined. This experiment was repeated with similar results. Each point represents the mean of three determinations ± SEM.

the insulin present. In most studies carried out so far DCC-treated serum was used, which contains biologically active insulin and IGFs [12]. The level of these growth factors, however, will be serum batch-dependent. This may account for the fact that, although mostly high concentrations of progestins have been reported to inhibit E2-induced proliferation of hormone-dependent breast cancer cells [6, 9], some authors failed to find such an effect [28, 29].

The absence of a stimulatory effect at low concentrations is not due to the progestins used being weak ligands since they all bind with high affinity to the PR of MCF7 cells ([30], van der Burg *et al.*, unpublished). The high concentrations of progestins necessary to stimulate proliferation during the 4 day period of the growth experiments do not seem to be caused by degradation of the progestins. In the first place, the same dose–response curves were found in the much shorter [^{3}H]thymidine incorporation experiments. A further indication that these stimulatory effects are not caused by low concentrations of progestins stimulating the PR, is suggested by the fact that antiprogestins were not able to block this effect. Moreover, the progestins MPA and Org 2058 did not stimulate MCF7 proliferation.

The fact that high concentrations of progestins are needed for growth merely suggests that it is not mediated via the PR, but rather through a crossreaction with another receptor. Since this effect could be blocked almost completely by antiestrogens (and not by antiglucocorticoids or antiprogestins), it is probably caused by crossreaction with the ER. Although gestodene has been reported to bind to the ER in breast cancer tissue [31], no further evidence for direct ER binding has been found for the synthetic progestins that we used ([30, 32], H. J. Kloosterboer, personal communication). Therefore, metabolites of the compounds are more likely to be responsible for the observed E2-like effects. In this respect, it is interesting to note that all stimulatory compounds possess the 17β-OH group that is important for E2 activity, whereas Org 2058 and MPA do not. Therefore, metabolites with a reduced A-ring (e.g. by aromatization) of the former, but not the latter compounds may have estrogenic activity. Aromatase activity is a common phenomenon in human breast cancer cells [33], and a low level of aromatization has also been found in MCF7 cells [34, 35]. However, a low level of conversion of the 10^{-7}–10^{-6} M concentrations of progestins to an estrogenic compound would be sufficient to stimulate MCF7 cells, since these cells are half-maximally stimulated by approx. 10^{-11} M concentrations of E2 [12]. Evidence for crossreaction of norgestrel that may be caused by its metabolites, with the ER of the human breast cancer cell line ZR-75-1 has recently been found [3]. The observation that the progestin stimulation was potentiated by low concentrations of insulin strengthens the notion that it is ER-mediated, since a similar synergistic effect of insulin and E2 is observed in stimulating MCF7 cells [12]. A stimulatory effect of more physiological concentrations of R5020 (10^{-8} M) on T47D cells has been reported [9], but it is not known which receptor mediates this effect.

It has been suggested that stimulation of the insulin receptor may lead to growth stimulatory effects of progestins at more physiological concentrations [7, 11]. However, at physiological concentrations of progesterone, the up to 3-fold increase of insulin receptors is paralleled only by a small increase in the response to insulin ([36, 11], this study). This is in line with the finding that insulin stimulates MCF7 and T47D cells mainly via crossreaction with the IGF-I receptor [37–40], and argues against an important role in this respect for the insulin receptor itself, as was found in a recent study [41].

Gestodene has been reported to bind to specific gestodene binding sites while other progestins, such as 3-ketodesogestrel, do not compete for this binding [5]. These gestodene bindings sites were suggested to be potentially important to the inhibitory action of gestodene towards breast cancer cells. Our results, that show that both gestodene and 3-ketodesogestrel have a nearly identical biological activity towards breast cancer cells, do not support such a role for the gestodene binding sites. Recently, Colletta *et al.* [4] showed growth inhibition of MCF7 cells by gestodene, and to a much lesser extent by other progestins, in the absence of E2. This contrasts with our data and literature data [3, 7, 8] that do not show inhibition of proliferation in the absence of E2 by all progestins, including gestodene. We do not know the reason for this discrepancy. A difference between our study and the study of Colletta *et al.* [4] is that they used a 3-week period to deplete the cells during which the PR will be downregulated [24] and proliferation will cease almost completely ([16], van der Burg

et al., unpublished), while we used a short steroid depletion protocol that prevented extensive receptor downregulation.

In general, a situation in which no E2 is present will not occur in oral contraceptive users. Therefore, we do not think that effects on breast tumor cells under these conditions, either inhibitory [4] or stimulatory [3, 7, 8] are relevant. At pharmacological concentrations these compounds may have estrogenic activity. However, in oral contraceptive users the serum levels of the synthetic progestins are low [42], and eventual estrogenic effects will give an insignificant contribution to those caused by estrogens from endogenous sources and the contraceptives themselves. The absence of either stimulatory or inhibitory effects of the progestins on breast tumor cells at physiological concentrations does not support a role for these compounds in directly influencing hormone-dependent breast cancer.

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