J. Steroid Biochem. Molec. Biol. Vol. 38, No. 3, pp. 345–350, 1991
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0960-0760/91 $3.00 + 0.00
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PROLIFERATION OF GUINEA-PIG UTERINE EPITHELIAL CELLS IN SERUM-FREE CULTURE CONDITIONS: EFFECT OF 17β-ESTRADIOL, EPIDERMAL GROWTH FACTOR AND INSULIN

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(Received 31 July 1990)

Summary—The effects of 17β-estradiol (E_2), epidermal growth factor (EGF) and insulin, alone or in association on guinea-pig uterine epithelial cell proliferation were examined in serum-free culture conditions. Primary cultures of epithelial cells were made quiescent by serum depletion, then incubated in a chemically defined medium. In this medium, insulin increased DNA synthesis but not in a dose-dependent manner for concentrations ranging from 0.2 to 10 μg/ml. A significant effect of EGF was found only for the highest concentration tested (100 ng/ml). E_2 alone or in the presence of insulin (1 μg/ml) had no effect whatsoever on the concentration tested (10^{-10}–10^{-5} M). Insulin (10 μg/ml) plus EGF (100 ng/ml) exerted on DNA synthesis and cell proliferation a significant additive effect which was identical to the growth stimulation induced by 10% fetal calf serum. The effects of insulin plus EGF were not modified by the addition of E_2. These findings suggest that E_2 is not directly mitogenic for uterine epithelial cells in defined culture conditions and that the mitogenic response to optimal concentration of insulin plus EGF is independent of E_2.

INTRODUCTION

The patterns of epithelial cell proliferation in guinea-pig uterus is correlated with the cyclic variations of circulating 17β-estradiol (E_2) and progesterone (P) during the estrous cycle [1]. Furthermore, in ovariectomized guinea-pig estrogen administration exerts an uterotropic effect [2], increases epithelial cell proliferation [3] and is associated with morphological and functional differentiation of the uterine epithelium [4]. Uterine guinea-pig epithelial cells thus provide a suitable system on which to study the hormonal regulation of cell proliferation. The mechanism by which estrogens affect cellular proliferation in normal cells is not fully understood. Although estrogens have been shown to stimulate *in vivo* the growth of uterine, vaginal and mammary cells in various species [5–7], the extent of stimulation observed *in vitro* is variable and depends on experimental conditions. *In vitro*, estrogens do not stimulate mouse uterine epithelial [8] or vaginal [9] cell proliferation in a defined medium. In the same conditions, however, rabbit uterine epithelial cell growth is enhanced by estrogens only when cultured at low density [10]. Conversely, estrogens stimulate the growth of human uterine cells cultured in the presence of a steroid-free calf serum [11]. These discrepancies may result from the inability of estrogens to induce cell proliferation in already optimized media in the absence of seric factors [8].

It has been recently reported that the estrogen responsiveness of uterine cells, *in vitro*, is dependent upon the presence of stromal cells [12, 13]. These observations support the hypothesis that estrogens act indirectly on cell proliferation via autocrine and/or paracrine growth factors including epidermal growth factor (EGF) and insulin related polypeptides [14–18]. To study, *in vitro*, the action of E_2, EGF and insulin on uterine epithelial cell proliferation, it is thought that it is of interest to use culture conditions that favour the yield of an homogeneous population and the growth of cells. In our laboratory, we have developed a primary culture system for guinea-pig uterine epithelial cells [4, 19] and their hormone responsiveness has been recently demonstrated [20, 21].

In the present study, guinea-pig uterine epithelial cells were grown *in vitro* then made quiescent by serum deprivation and further

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incubated in a chemically defined medium in which their mitogenic response to E_2, insulin and EGF were compared.

MATERIALS AND METHODS

Materials

RPMI 1640 Medium with or without Phenol Red, Phenol Red-free Ham's F12, L-glutamine, penicillin, streptomycin, fungizone, Hepes buffer and fetal calf serum (FCS) were obtained from Vietech (St Bonnet de Mure, France). Mouse EGF, bovine insulin, E_2, cortisol (F), bovine serum albumin (BSA), transferrin (TF), sodium selenite and retinol acetate were purchased from Sigma Chimie (La Verpillière, France). Worthington collagenase CLS I (284 U/mg) was obtained from InterMed (Strasbourg, France). [Methyl-^{3}H]thymidine (40–60 Ci/mmol) was obtained from Amersham (Les Ulis, France). Other chemicals were of analytical grade. E_2 and F were prepared from (10^{-2}–10^{-4} M) stock solutions in 100% ethanol, then diluted to a 10^{-5} M working stock in sterile Ham's F12 medium (final concentration of ethanol in the solution was less than 0.1%). Retinol acetate was prepared as concentrated stock solution (0.1%, w/v) in dimethylsulfoxide.

Cell culture

The method for the isolation of glandular organoids from guinea-pig endometrium and the epithelial cell culture procedure were those of Chaminadas *et al.* [19] modified by Alkhalaf *et al.* [4]. Organoids consisting in glandular epithelial cells (4.10^2 epithelial cell clusters per 60 mm dish) were plated in 7 ml growth medium: RPMI 1640 with Phenol Red supplemented with 10% FCS, L-glutamine (4 mM), EGF (25 ng/ml), insulin (1 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml) and Hepes buffer (20 mM). The dishes were incubated in a humidified atmosphere composed of 5% CO_2 and 95% air and the growth medium was changed after 2 days. The cells reached approx. 75% confluence within 3–4 days. After an appropriate growth time, the cells were made quiescent by depressing the serum concentration of the medium [22]. The cells were washed with 5 ml of low serum medium (LSM) composed of Phenol Red-free RPMI 1640 supplemented with 1% of heated and dextran-charcoal coated stripped FCS (DCC-FCS), L-glutamine (4 mM), insulin (1 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml) and Hepes buffer (20 mM). This medium was changed every 16 h for 2 days. At the end of the procedure, the quiescent cells were screened after staining with the fluorochrome Hoechst 33258 (Intermed, Strasbourg, France) and found free of mycoplasma contamination. The percentage of epithelial cells was determined by staining with an anticytokeratin No. 18 monoclonal antibody (Biosoft, Paris, France). Seventy five to 95% of quiescent cells were immunostained. Cell viability assessed by the trypan blue exclusion test was more than 95%.

To study the effect of insulin, EGF and E_2 on DNA synthesis in the quiescent cells, LSM were changed for 5 ml of a chemically defined medium (CDM). CDM was composed of Phenol Red- and thymidine-free Ham's F12 with L-glutamine (4 mM), F (10^{-6} M), TF (10 μg/ml), retinol acetate (100 ng/ml), sodium selenite (10 μg/ml), BSA (1 mg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), fungizone (2.5 μg/ml) and Hepes buffer (20 mM). Quiescent cells were incubated in CDM for 16 h.

DNA synthesis

DNA synthesis was measured by [^{3}H]-thymidine incorporation into acid insoluble fraction. [^{3}H]thymidine (3 μCi/ml) was added to the culture medium for a pulse labelling of 1 h at the end of the incubation periods. The cell layer was then gently washed three times with 5 ml cold phosphate buffer saline (0.1 M) at 4°C containing a 200-fold excess of unlabelled thymidine. The cells were harvested by trypsination [0.1% (w/v) trypsin and 0.5 mM EDTA] and separated into 2 fractions: one was used for cell counting with a haemocytometer, the other to determine [^{3}H]thymidine incorporation in the acid-insoluble fraction. Trichloracetic acid was added at a final concentration of 5% (w/v) for 15 min, and the precipitate was washed twice with absolute ethanol. Precipitates were then dissolved in 1 ml of 0.2 M NaOH, and 500 μl of each sample were counted for radioactivity after neutralization by HCl in 6 ml of a liquid scintillation solution (Ready Value, Beckman, Strasbourg, France).

RESULTS

Effects of insulin, EGF and E_2 on DNA synthesis

The effects of increasing concentrations of insulin and EGF on DNA synthesis were tested

in quiescent cells incubated with CDM for 16 h. DNA synthesis was monitored by [^{3}H]thymidine incorporation (1 h pulse labelling at the end of the incubation period) and the results were expressed in relation to control quiescent cells incubated without growth factors (Fig. 1). [^{3}H]thymidine incorporation value in the control cells was 42.4 ± 2.8 dpm/10^3 cells (mean $\pm$ SEM for 5 separate dishes). DNA synthesis was higher in insulin-treated cells than in untreated controls. However, the experimental curve rapidly reached a plateau without significant variation of DNA synthesis for concentrations of insulin ranging from 0.2 to 10 μg/ml. Compared with untreated control cells no significant variation of DNA synthesis was observed in EGF-treated cells except for the highest tested concentration (100 ng/ml) which increased significantly DNA synthesis as compared with controls.

To study the effect of E_2, the quiescent cells were incubated for 16 h with CDM containing increasing concentrations of E_2 (0.1 nM–10 μM). [^{3}H]thymidine incorporation into DNA was monitored by a 1 h-pulse labelling at the end of the incubation period (Fig. 2). No statistically significant variation of [^{3}H]-thymidine incorporation (ANOVA: $F = 1.40$, $P > 0.05$) occurred with increasing concentrations of E_2. The lack of E_2 action may be due to the absence of insulin as reported by Van der Burg *et al.* [23]

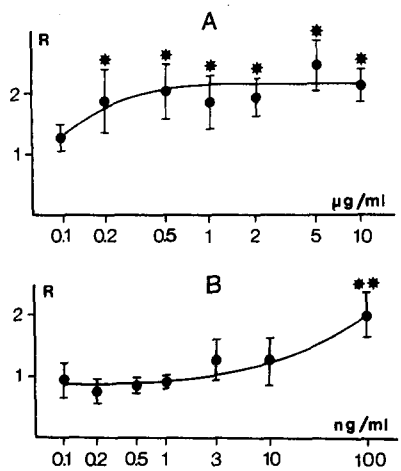

Fig. 1. Effects of increasing concentrations of insulin and EGF on DNA synthesis. Quiescent epithelial cells were incubated in the chemically defined medium for 16 h with insulin (A) or EGF (B). DNA synthesis was monitored by 1 h pulse labelling with [^{3}H]thymidine (3 μCi/ml) at the end of the incubation period. Untreated control cells was simultaneously processed. R: ratio of [^{3}H]thymidine incorporation into stimulated cells to control cells (control cells: 42.4 ± 28.0 dpm/10^3 cells; mean $\pm$ SEM; $n = 5$). *$P < 0.05$ vs control; **$P < 0.01$ vs control (Mann–Whitney U-test).

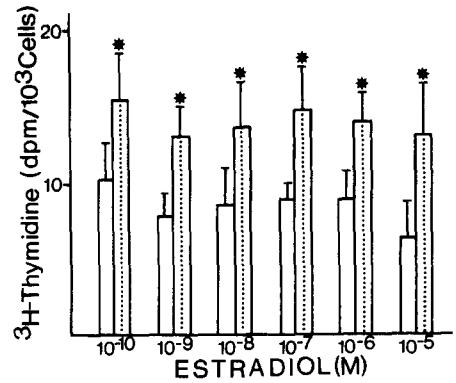

Fig. 2. Effect of increasing concentrations of 17β-estradiol on DNA synthesis. Quiescent epithelial cells were incubated with the chemically defined medium for 16 h with increasing concentrations of 17β-estradiol alone (open columns) or in combination with insulin (1 μg/ml) (dotted columns). DNA synthesis was monitored by 1 h pulse labelling with [^{3}H]thymidine (3 μCi/ml) at the end of the incubation periods. Data are the mean values $\pm$ SEM of three separately treated dishes. *$P < 0.05$ as compared with 17β-estradiol alone (Mann–Whitney U-test).

for MCF-7 cells. The action of E_2 was thus tested in the presence of insulin (1 μg/ml). A significant increase of [^{3}H]-thymidine incorporation was induced by E_2 plus insulin as compared with experiments conducted with E_2 alone. However, in the presence of insulin, no dose-effect of increasing concentration of E_2 was observed (ANOVA: $F = 0.34$; $P > 0.05$).

To test a possible additive effect of E_2 and growth factors on DNA synthesis, quiescent cells were incubated for 16 h with CDM containing either E_2 or insulin or EGF alone or in combination as indicated on Fig. 3. Untreated control cells and cells incubated in CDM supplemented with 10% FCS were simultaneously processed. DNA synthesis was monitored by 1 h-pulse labelling with [^{3}H]thymidine. E_2 alone had no effect on DNA synthesis as compared with untreated control cells. Insulin or EGF alone induced a significant increase in [^{3}H]-thymidine incorporation. A significant additive effect was found when cells were incubated with insulin plus EGF. This effect was not enhanced by the addition of E_2 and was equivalent to that exerted by 10% FCS.

Effect of insulin, EGF and E_2 on cell proliferation

In order to study the effect of growth factors and E_2 on cell proliferation, uterine epithelial cells were cultured in multi-well plates (10 mm diameter) instead of dishes. Quiescent cells were obtained using the same procedure as described in the "Materials and Methods" section. The

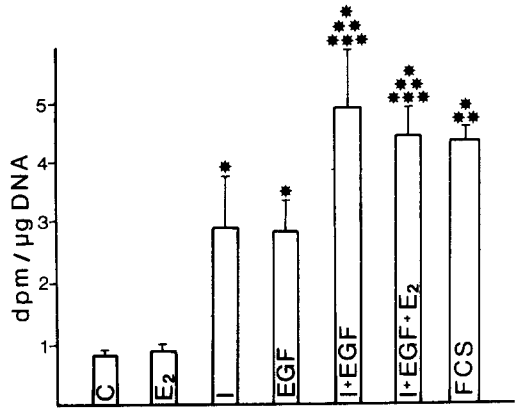

Fig. 3. Effect of 17β-estradiol (E_2), insulin (I) and epidermal growth factor (EGF) on DNA synthesis. Quiescent epithelial cells were incubated in the chemically defined medium for 16 h with E_2 (10^{-8} M) or I (10 µg/ml) and EGF (100 ng/ml) alone or in combination. Untreated control cells (C) and cells incubated with 10% fetal calf serum (FCS) were simultaneously processed. DNA synthesis was monitored by 1 h pulse labelling with [^{3}H]thymidine (3 µCi/ml). Data are the mean values ± SEM of 5 separately treated dishes. *$P < 0.05$ as compared with 17β-estradiol; **$P < 0.05$ as compared with I or EGF; ***$P > 0.05$ as compared with 10% FCS (Mann–Whitney U-test).

initial number of quiescent cells per well was measured on 4 randomly chosen wells: $87.3 \times 10^3 \pm 12.5 \times 10^3$ cells/well (mean ± SEM). Quiescent cells were then cultured for 90 h in CDM (0.5 ml) supplemented with either insulin (10 µg/ml) plus EGF (100 ng/ml) or both growth factors in combination with E_2 (10^{-8} M). Untreated control cells and cells incubated in the presence of 10% FCS were simultaneously processed. The number of control cells did not vary during the time-course of the culture compared with the initial number of cells. When quiescent cells were incubated with insulin or EGF alone, a slight but no significant increase ($P > 0.05$) of the cell number was observed compared with untreated control cells (data not shown). Incubation of quiescent cells with insulin plus EGF induced a significant increase of the cell number as compared with untreated control cells (Fig. 4). When E_2 was combined with insulin plus EGF no additive effect was observed. The effect of both growth factors with or without E_2 was identical to that observed with 10% FCS.

DISCUSSION

The action of E_2 and growth factors i.e. insulin and EGF on the proliferation of guinea-pig uterine epithelial cells grown in primary culture were examined. At the end of the initial growth phase, the hormone responsiveness of the growing cells was assessed on the basis of the presence of estrogen and progesterone receptors (PR) and of the increase of the PR by E_2 stimulation [19]. Progesterone added to growing cells primed with E_2 induced a significant increase of estrogen sulfotransferase activity, a marked decrease in nuclear PR content and the synthesis of specific proteins [21]. Growing cells were made quiescent by depressing the serum concentration in the medium for 2 days. The choice of a medium supplemented with 1% DCC-FCS was essentially dictated by the fact that this medium did not affect cell viability and allowed the adhesion of the quiescent cells to the plastic surface of the dishes. During the period of serum depletion, the cell number did not vary and DNA synthesis was significantly reduced [22]. Incubation of quiescent cells for 48 h in LSM did not alter their ability to re-enter the cell cycle and to proliferate after stimulation by an unspecific mitogen i.e. 10% FCS [22].

When quiescent cells were incubated in CDM, E_2 alone was unable to induce DNA synthesis whatever the concentration used (10^{-10}–10^{-5} M). The inability of estrogens to directly induce *in vitro*, cell proliferation has been reported by Imagawa *et al.* [24] for mammary epithelial cells, by Tomooka *et al.* [8] for uterine epithelial cells and by Uchima *et al.* [9] for vaginal epithelial cells. However, in a synthetic medium, Gerschenson *et al.* [10] demonstrated that E_2 induced the proliferation of rabbit uterine epithelial cells grown in primary culture only in low density cultures. The lack of E_2 action

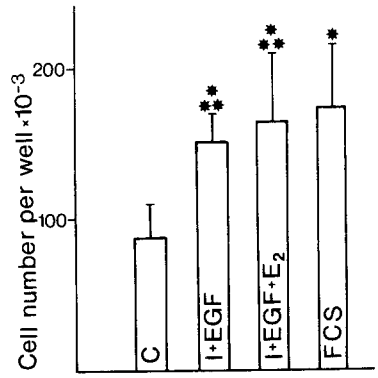

Fig. 4. Effects of insulin (I) plus EGF and 17β-estradiol (E_2) on the proliferation of quiescent epithelial cells. Cells were incubated in the chemically defined medium (CDM) for 90 h with I (10 µg/ml) plus EGF (100 ng/ml) or I plus EGF plus E_2 (10^{-8} M). Untreated control cells (C) and cells cultured in the CDM supplemented with 10% fetal calf serum (FCS) were simultaneously processed. Data are the mean values ± SEM of 5 separately treated wells. *$P < 0.02$ as compared with controls; **$P > 0.05$ as compared with 10% FCS (Mann–Whitney U-test).

observed in the present study was not due to cell culture density since the hormonal effect was tested in low density conditions and insulin plus EGF induced a significant cell proliferation. Another possibility is the loss of estrogen receptors during cell culture. We have already reported that estrogen receptors were present in cultured epithelial cells and that the level of progesterone receptors was increased by E_2 stimulation [19]. These observations confirm earlier findings [25] showing that the effects of E_2 on cell proliferation and on progesterone receptors can be dissociated. A paracrine stimulation of epithelial cells by stromal cells may be inferred from the fact that mouse uterine epithelial cells stimulated by E_2 proliferated *in vitro* only when they were cocultured with stromal cells [13]. In our system, the lack of E_2 action may be due to the fact that cell cultures were conducted using preparations enriched with epithelial cells and the residual number of non epithelial cells (5–25% of the cell population) was not sufficient to sustain a paracrine effect. Studies now in progress in our laboratory are being conducted with mixed cultures of stromal and epithelial cells in CDM.

Converging results support the hypothesis that the proliferative response to estrogens is mediated via growth factors. Insulin-related polypeptides [15] and EGF [14, 16] are considered to be potential mediators of estrogen-induced proliferation. In quiescent epithelial cells, insulin increased DNA synthesis but not in a dose-dependent manner for concentrations higher than 0.2 μg/ml. A significant effect of EGF was found only for the highest concentration tested (100 ng/ml). Additive effect was found when both growth factors were added in combination. This effect was identical to that obtained on DNA synthesis and cell proliferation with 10% FCS. Estrogen and insulin synergism has been reported *in vitro* by Toran-Allerand *et al.* [26] for neurites and by Van der Burg *et al.* [23] using a suboptimal concentration of insulin for MCF-7 cells. A low concentration of insulin (1 μg/ml) increased DNA synthesis of quiescent epithelial cells, but this effect did not vary with increasing concentration of E_2.

In the chemically defined medium, the same amount of stimulation of DNA synthesis and cell proliferation was obtained with either EGF plus insulin or both growth factors plus E_2 or 10% untreated FCS. These results demonstrate the positive influence *in vitro* of EGF and insulin on epithelial cell growth and the lack of estrogen effect in serum-free conditions either in the absence or in the presence of optimal concentrations of growth factors. However, *in vivo*, exogenous E_2 stimulated mitoses in ovariectomized guinea-pig uterine epithelial cells [3]. These observations support the hypothesis of an indirect action i.e. paracrine action of estrogen on epithelial cells. Growth factors acting on uterine epithelial cell proliferation may be secreted by other cell types under estrogen stimulation [27].

In conclusion, our data show that: (i) an additive effect exists between EGF and insulin on the stimulation of epithelial cells proliferation in a chemically defined medium; (ii) E_2 *per se* has no effect on epithelial cell proliferation in defined conditions; (iii) the mitogenic response of uterine epithelial cells to optimal concentrations of EGF and insulin is not influenced by E_2 and; (iv) the response to EGF and insulin in serum-free medium is equal to the mitogenic response of epithelial cells to 10% FCS. This observation indicates that our serum-free culture medium is suitable for the study of the mechanism by which growth factors and estrogen regulate normal epithelial cell proliferation.

Acknowledgements—The authors wish to thank B. Montenoise for technical assistance, M. David and C. Ferniot for manuscript typing and M. Gill for reviewing the English. This work was supported by the Conseil Régional de Franche-Comté and by a grant from the Association pour la Recherche sur le Cancer.

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