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Journal of Steroid Biochemistry and Molecular Biology 70 (1999) 133–142

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Regulation of cell growth of a progestin-dependent murine mammary carcinoma in vitro: progesterone receptor involvement in serum or growth factor-induced cell proliferation

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Received 26 October 1998; accepted 7 June 1999

Abstract

Primary cultures of the medroxyprogesterone acetate-induced mouse mammary tumor line C4-HD are stimulated by medroxyprogesterone acetate (MPA) or progesterone. Serum obtained from ovariectomized, MPA-treated animals (OVX-MPA) exerts a stimulatory effect that is significantly higher than that induced by serum obtained from OVX mice with the exogenous addition of MPA, suggesting the involvement of MPA-induced serum factors potentiating the proliferative effect of MPA. The object of this paper is to further explore the stimulatory effect of mouse serum and to investigate the role of aFGF and bFGF on cell proliferation. The role of PR as possible mediators was tested using two different antiprogestins and antisense oligodeoxynucleotides of PR A isoform. Serum was obtained from OVX untreated or MPA-treated mice and was charcoalized and/or heat-inactivated. The effect of MPA or mifepristone at 10 nM concentrations was tested. Charcoalization and heat inactivation exerted a stimulatory effect ($P < 0.01$) when OVX-serum was used. This effect was potentiated by MPA. Charcoalized OVX-MPA serum induced a significant inhibition of cell proliferation that was restored by the exogenous addition of MPA or by heat inactivation. Mifepristone induced an inhibition of ^{3}H-thymidine uptake when OVX-MPA serum was used. These results suggest that serum factors activated by different manipulations may replace the stimulatory effect of MPA. When charcoalized fetal calf serum (chFCS) was used, a higher proliferative activity was obtained using higher serum concentrations. Mifepristone and onapristone 10 nM also inhibited this effect. aFGF and bFGF 100 ng/ml were both able to stimulate ^{3}H-thymidine uptake. MPA exerted an additive effect. Mifepristone 10 nM inhibited bFGF and MPA + bFGF induced cell proliferation. Antisense oligodeoxynucleotides of PR (ASPR) were used to further confirm the participation of PR in the proliferative pathway of these cells. They inhibited serum and bFGF-induced cell proliferation in a specific dose-dependent manner. Our results suggest that PR play a central role in proliferation and suggest the existence of a cross-talk between steroid and growth factor signaling pathways.

Keywords: Progesterone receptors; Mammary adenocarcinomas; Fibroblast growth factors; Antiprogestins; Antisense

1. Introduction

The role of progestins in mammary tumor cell proliferation remains a controversial issue [1,2], but several experimental data point towards a possible role as stimulators [1]. Estrogens induce cell proliferation in mammary gland and other target tissues, but the intimate mechanism involved has not yet been completely elucidated. It has been suggested that their actions may be indirect, and many different hypotheses ranging from the activation of growth factors [3] to the inhibition of estrocolyones [4] have been put forward. Their effects may also be mediated by the induction of the synthesis of PR and by the induction and release of adenohypophyseal hormones [5].

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0960-0760/99/$ - see front matter
PII: S0960-0760(99)00108-9

We have developed an experimental model in which ductal progestin-dependent (PD) mammary adenocarcinomas are induced by the continuous administration of medroxyprogesterone acetate (MPA) to BALB/c female mice; these tumors express high levels of estrogen (ER) and progesterone receptors (PR) [6–9]. Primary epithelial cultures are stimulated by MPA, an effect that seems to be mediated by PR [10]. In vitro tumor cell proliferation can also be stimulated to levels similar to those obtained with MPA, using normal serum obtained from in vivo MPA-treated ovariectomized mice (OVX-MPA). Surprisingly, only a very slight increase in cell growth was detected when MPA was added to normal serum obtained from ovariectomized mice (OVX) that had not been treated with the hormone. These results suggested the existence of other serum factors, probably induced by MPA, which were able to potentiate its stimulatory effect [10]. In this paper we have further explored the proliferative actions of these putative serum factors on MPA-induced proliferation using heat-inactivated and/or steroid-stripped mouse serum obtained from control or OVX-MPA animals. We demonstrate that 5% charcoalized-heat inactivated serum exerts an intrinsic proliferative activity and that this effect can be abolished by antiprogestins, suggesting that PR may be involved in this pathway.

As part of an ongoing project in our lab, in which we are evaluating the effects of different growth factors [11,12], we have also focused our interest on the role of the FGFs in the proliferation of epithelial and stromal cells from PD mammary tumors. The FGFs are a family of angiogenic growth factors expressed in both normal and malignant mammary tissue [13]. They are potent mitogens that play a significant role in stromal-epithelial interactions [14]. In this report we also demonstrate that both aFGF and bFGF induce epithelial cell proliferation in culture, and that this effect can be prevented by mifepristone or antisense oligodeoxynucleotides of PR (ASPR), suggesting that the PR may also be activated by non-steroidal factors.

In this paper we provide evidence that in our experimental model, the PR is an essential regulatory mechanism for tumor cell proliferation, through which several pathways may interact.

2. Materials and methods

2.1. Animals

All experiments were carried out using 2-month-old virgin female BALB/c mice raised at the National Academy of Medicine, Buenos Aires. They were housed 3–6 per cage in air conditioned rooms at $20 \pm 2^{\circ}C$, kept under an automatic 12 h light/12 h darkness schedule and given pellets and tap water ad libitum. Animal care was in accordance with institutional guidelines.

2.2. Tumors

The experiments were carried out using a PD tumor line (C4-HD), originated in an MPA-treated BALB/c mouse in 1985, which is maintained by in vivo serial syngeneic transplants in MPA-treated mice [7]. This tumor expresses high levels of ER (Kd: 2.3 ± 1 nM, 258 ± 67 fmoles/mg prot, $n = 4$) and PR (Kd: 9.4 ± 3.7 nM, 719 ± 289 fmoles/mg prot, $n = 4$) which are maintained in primary cultures (49.7 ± 9.3 fmoles/10^5 cells) [10]. Tumor stromal cells, which lack PR [10], were used as a control in experiments using ASPR.

2.3. Cell lines

In control experiments we used MCF-7 cells provided by Dr I. Luthy (IBYME, Buenos Aires) and NMuMG cells provided by Dr J.C. Calvo (IBYME, Buenos Aires). The MCF-7 cell line was derived from a pleural effusion of a patient with breast cancer and is estrogen responsive. PR expression is under estrogen regulation and progesterone does not seem to be directly implicated in cell proliferation [15]. NMuMG is an epithelial cell line derived from normal mouse mammary gland; the expression of either PR or ER has not been reported [16].

2.4. Hormones, growth factors and antihormones

MPA, EGF, aFGF and bFGF were obtained from Sigma Chem. Co., St Louis MO, USA. Mifepristone was a gift of Roussel Uclaf, Romainville, France and onapristone was a gift of Schering, Germany. MPA, onapristone and mifepristone were dissolved in absolute ethanol at 10^{-3} M (stock solution). Growth factors were dissolved in medium according to the instructions of the manufacturer. Working solutions were freshly prepared before each experiment to a final ethanol concentration of less than 0.1% for steroids.

2.5. Use of antisense oligodeoxynucleotides of PR

The antisense oligomers of PR used in this study, previously used by Mani et al. [17] in in vivo studies in rats, were modified to reflect the sequence of the mouse PR [18]. It includes the initial codon of the PR A isoform. ASPR (5′-ACTCATGAGCGGGGACA-ACA-3′) and the scrambled sequence (ScPR) (5′-ACGCTAGACTGACGACG AGA-3′) were synthesized by Cybersin PA and by Fagos SA, Buenos Aires, and purified by high pressure chromatography.

A NCBI BLAST search in the Gene Bank database revealed no ASPR homology higher than 70% with any known mouse gene.

2.6. Serum samples

One week after ovariectomy, 25 BALB/c mice were inoculated sc with 40 mg (0.2 ml) of MPA depot (Medrosterona, Gador Laboratories, Buenos Aires, Argentina). One week later they were bled through the eye along with control OVX animals ($n = 25$). Pooled blood was allowed to clot in sterile plastic tubes for 4 h and the sera were clarified by centrifugation. About 100 μl were obtained per animal. Some samples were heat-inactivated (56°C, 30 min), and/ or charcoalized. The sera were used directly after filtration or aliquoted and stored at −20°C for further use. Animals were anesthetized according to the NIH Guide for the Care and Use of Laboratory Animals.

2.7. Charcoal-adsorption

To strip the sera of steroids, charcoal (Merck) was added to fetal calf serum (FCS) or to mouse-serum to reach a final concentration of 0.05 mg/ml. Charcoal was maintained in suspension by shaking, and the extraction was carried out at 4°C overnight. Charcoal was removed by 5 consecutive centrifugations at 10,000 rpm for 15 min. In order to increase the efficiency of the procedure it was repeated twice, the second time for 3 h.

2.8. Culture media

DMEM/F12 (Dulbecco's modified Eagle's medium: Ham's F12, 1:1, without phenol red, 100 U/ml penicillin and 100 μg/ml Streptomycin). Fibroblastic medium: DMEM/F12 + 10% heat inactivated FCS (Gen SA, Buenos Aires) (FM). Washing medium: DMEM/F12 + 5% FCS (WM). Epithelial medium: DMEM/F12 + 5% chFCS (EM).

2.9. Primary cultures

Tumors were aseptically removed, minced and washed with DMEM/F12. The tissue was suspended in 5 ml of enzymatic solution (trypsin: 2.5 mg/ml, albumin: 5 mg/ml and collagenase type II (Gibco BRL): 850 U/ml in phosphate buffered saline (PBS)) and incubated at 37°C for 20 min, under continuous stirring. The liquid phase of the suspension was then removed and the undigested tissue incubated for an additional 20 min with fresh enzymatic solution. Enzyme action was stopped by adding WM. Epithelial and fibroblastic cells were separated by a modification of the sedimentation technique described previously [10]. Briefly, the cell suspension obtained was resuspended in 15 ml of FM and allowed to sediment for 20 min. The upper 5 ml, corresponding to the fibroblastic fraction, was seeded in flasks, and the cells were allowed to attach during 1–2 h after which the medium containing unattached cells was removed and replaced by fresh FM. The sedimented cells correspond to the epithelial enriched fraction, which was resuspended again in 15 ml of WM and allowed to sediment for another 20 min. The upper 15 ml were discarded and this procedure was repeated 10 times more or less until no fibroblasts were detected in the supernatant. Cells were plated in culture flasks with EM and allowed to attach for 24–48 h. The medium was then removed and replaced by fresh medium containing 10^{-8} M MPA. This medium was changed every 2–3 days. At confluence, or when cell clusters looked overcrowded, the cells were detached with 0.25% trypsin, washed, and resuspended in fresh EM. The resulting suspension was used in the different assays.

2.10. ^{3}H-Thymidine uptake assay

In a Corning 96-well microplate, 0.1 ml/well of a cell suspension were seeded in EM at a concentration of 10^5 cell/ml. After attachment (24–48 h), the cells were incubated for 48 h with the experimental solutions to be tested. Half the media was changed every 24 h. The cells were incubated with 0.4 μCi of ^{3}H-thymidine (specific activity: 20 Ci/mmol) for 24 h, trypsinized and harvested in a cell harvester. Filters were counted in a liquid scintillation counter. Assays were performed in octuplicates and mean and standard deviation were calculated for each solution tested. The differences between controls and experimental groups were analyzed by ANOVA followed by Dunnet's T test between groups.

2.11. Progesterone receptor binding

The ability of ASPR to inhibit PR was tested by binding techniques. Cells were incubated with or without 20 μg/ml of ASPR in the same experimental conditions used in the experiments of cell proliferation and PR were evaluated using the whole cell method as previously described [10]. Cells were counted using Neubauer chambers and receptor values were expressed as fmoles/10^5 cells. The percentage of inhibition in each assay ($n = 3$) was evaluated.

3. Results

Vimentin and cytokeratin immunocytochemical staining was used to rule out cross contamination in epithelial and fibroblastic cultures. Since only epithelial

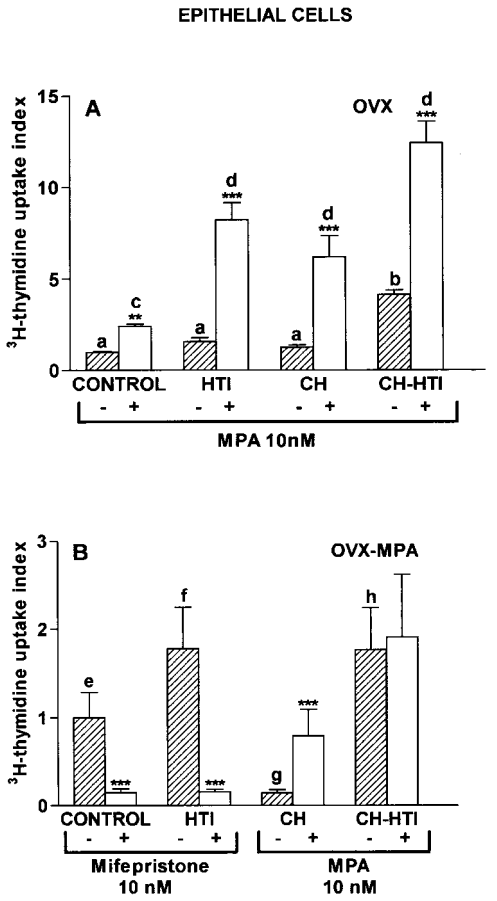

Fig. 1. Thymidine uptake in epithelial cells cultured in the presence of 5% serum obtained from ovariectomized untreated mice (OVX) (A) or treated for 1 week with MPA (OVX-MPA) depot 40 mg sc (B). Serum was used either untreated, charcoalized (CH) and/or heat inactivated (HTI). The cells were incubated in the absence or presence of MPA 10 nM or mifepristone 10 nM, for 48 h. The ^{3}H-thymidine index is the ratio between the total cpm (mean $\pm$ SD) counting of each experimental group ($n = 8$) and the control value obtained using serum from untreated OVX mice (503 ± 83 cpm) (A) or MPA-treated mice (3244 ± 928) (B). $^{**}P < 0.01$ treated vs untreated, $^{***}P < 0.001$ treated vs untreated, a and c vs b: $P < 0.05$, c vs d: $P < 0.001$, e vs f: $P < 0.05$, e, f and h vs g: $P < 0.01$. This is a representative experiment of three others.

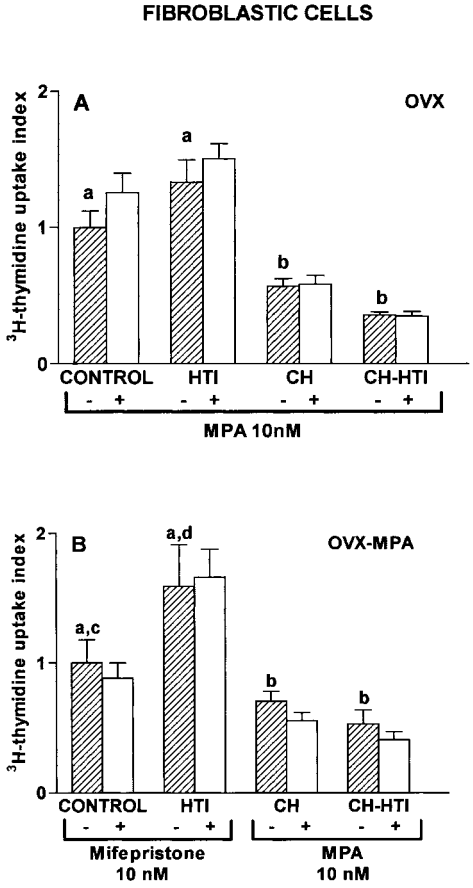

Fig. 2. Thymidine uptake of fibroblastic cells cultured in the presence of 5% serum obtained from ovariectomized mice untreated (OVX) (A) or treated for 1 week with MPA (OVX-MPA) depot 40 mg sc (B). Serum was used either untreated, charcoalized (CH) and/or heat inactivated (CH-HTI, HTI). The cells were incubated in the absence or presence of MPA 10 nM or mifepristone 10 nM, for 48 h. The ^{3}H-thymidine index is the ratio between the total cpm counting (mean $\pm$ SD) of each experimental group ($n = 8$) and the control value obtained using serum from OVX untreated mice (6296 ± 1700 cpm) (A) or MPA-treated mice (7304 ± 1308 cpm) (B). a vs b: $P < 0.001$, c vs d: $P < 0.05$. This is a representative experiment of three others.

cells are stimulated by MPA [10] and only fibroblasts by EGF [11], these controls were used whenever possible to reassure the absence of cross contamination between both cell types.

3.1. Mouse serum and MPA stimulatory activity

To further explore previous results in which we had demonstrated that serum obtained from MPA-treated mice could stimulate cell proliferation to values significantly higher than those obtained using serum from control mice with the exogenous addition of MPA, sera from different sources were exposed to various treatments. Charcoalized, heat-inactivated serum from OVX animals induced a significant increase in cell growth as compared with control serum, or with serum which was either charcoalized or heat-inactivated alone ($P < 0.05$) (Fig. 1A). This stimulatory activity proved to be even higher than that induced by serum from OVX mice with the exogenous addition of MPA ($P < 0.05$). The maximal proliferative activity was obtained when MPA was added to charcoalized, heat-inactivated serum.

Serum from MPA-treated OVX animals (OVX-MPA) also stimulated cell proliferation to values significantly higher than those obtained with serum from OVX mice with the addition of MPA ($P < 0.01$). Heat inactivation of OVX-MPA serum induced an increase in ^{3}H-thymidine uptake ($P < 0.05$). Charcoal treatment of OVX-MPA serum induced a significant decrease in ^{3}H-thymidine uptake ($P < 0.01$) which was

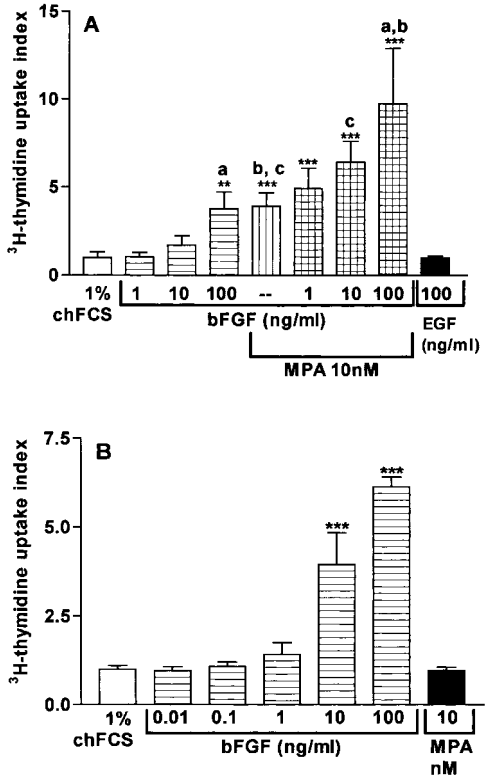

Fig. 3. Effect of bFGF on ^{3}H-thymidine uptake. (A) Epithelial cells were incubated with 1% chFCS with increasing concentrations of bFGF in the presence or absence of MPA 10 nM for 48 h. EGF was used to assess the absence of contaminant fibroblasts (see text). (B) Fibroblasts were incubated with 1% chFCS with increasing concentrations of bFGF for 48 h. MPA 10 nM was used to assess the absence of epithelial contaminants. These results are representative of four experiments. The ^{3}H-thymidine index is the ratio between the total cpm counting of (mean $\pm$ SD) each experimental group (n = 8) and the control (open bar, n = 16). $^{**}P < 0.05$ vs control, $^{***}P < 0.001$ vs control, a and b: $P < 0.001$, c: $P < 0.01$.

reverted by the exogenous addition of 1 nM MPA ($P < 0.001$) (Fig. 1B). Interestingly, when this serum was both charcoal-treated and heat-inactivated, it exerted a proliferative effect, similar to that induced by serum obtained from OVX-MPA mice. Mifepristone induced a significant decrease in the stimulatory efficiency in both (OVX-MPA) untreated or heat-inactivated.

The effects of these sera on fibroblast growth disclosed a pattern of ^{3}H-thymidine uptake different from that observed in epithelial cells (Fig. 2). All charcoalized sera had an inhibitory effect. Neither mifepristone nor MPA altered cell growth. Heat inactivation induced an increase in ^{3}H-thymidine uptake when OVX-MPA serum was used ($P < 0.05$).

When charcoalized and/or heat-inactivated FCS was used in similar experiments, the proliferative effects of MPA were also different according to the serum treatment used although the trend was not exactly the same. With chFCS in the presence of 10 nM MPA

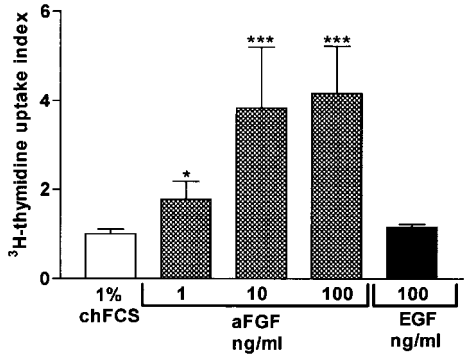

Fig. 4. Effect of increasing concentrations of aFGF on ^{3}H-thymidine uptake in the presence of 1% chFCS in epithelial cells. Cells were incubated for 48 h. EGF was used to assess the absence of contaminant fibroblasts (see text). The ^{3}H-thymidine index is the ratio between the total cpm counting (mean $\pm$ SD) of each experimental group (n = 8) and the control (n = 16, open bar). $^{*}P < 0.05$ vs control, $^{***}P < 0.001$ vs control. A representative experiment of other three is shown.

values of 15.764 ± 1525 cpm (n = 8) were obtained, while with ch-decFCS the values were of 8564 ± 958 cpm (n = 8), $P < 0.001$) under the same experimental conditions. In the absence of MPA the differences were less impressive: 5087 ± 641 cpm using chFCS and 4632 ± 792 cpm using ch-dec FCS.

There was a direct correlation between ^{3}H-thymidine uptake and the number of cells per well; in 10 nM MPA-treated cells a 1.739 ± 0.55 (n = 3) fold increase was calculated for thymidine uptake after an incubation period of 48 h, for the same treatment, cell counting yielded a 1.71 ± 0.06 (n = 3) fold increase, after 5 days of incubation.

These results indicate that in epithelial cells: (1) the degree of increase in cell proliferation is dependent on serum factors that may even replace the stimulatory activity of MPA; (2) MPA is able to increase this activity only when OVX serum is used, suggesting that systemic factors induced by MPA may also be potentiating its stimulatory activity; and (3) PR are mediating this proliferative effect. This is indicated by the fact that mifepristone abolishes OVX-MPA stimulatory activity. These effects are specific for epithelial cells since in fibroblasts charcoalization inhibits cell growth, suggesting that different mechanisms are regulating stromal cell proliferation.

3.2. Effect of FGFs on epithelial and fibroblastic cell growth

The effects of bFGF and aFGF in the presence of 1% chFCS were assayed in both epithelial and fibroblastic cultures. An increase in ^{3}H-thymidine uptake, similar to that obtained using 10 nM MPA was obtained with 100ng/ml of bFGF (Fig. 3A). An additive effect was observed when both agents were used

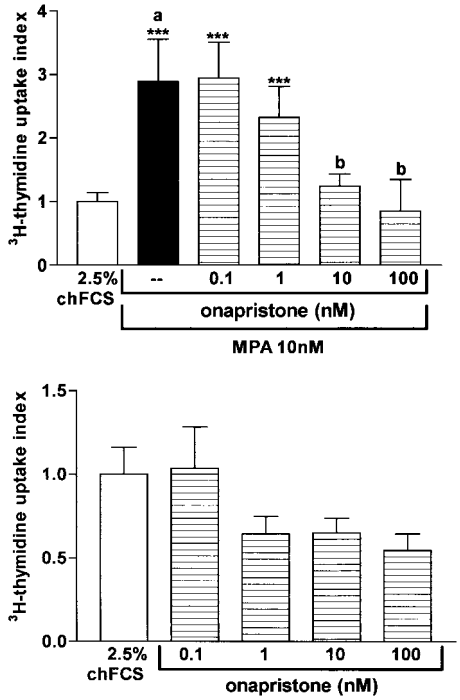

Fig. 5. Effect of onapristone on epithelial cell proliferation. The cells were grown with 2.5% chFCS in the presence (B) or absence (A) of 10 nM MPA. Cells were incubated for 48 h. The ^{3}H-thymidine index is the ratio between the total cpm counting (mean $\pm$ *SD*) of each experimental group ($n = 8$) and the control ($n = 16$, open bar). $^{***}P < 0.001$ vs control, a vs b: $P < 0.001$. A representative experiment of other three is shown.

($P < 0.05$). bFGF, in a similar range of concentrations, is also able to stimulate fibroblastic cell growth (Fig. 3B). The fact that EGF did not stimulate epithelial cell proliferation and MPA did not affect fibroblasts proliferation confirms that bFGF stimulates both types of cells. A similar proliferative effect was obtained when aFGF was used (Fig. 4), at concentrations even lower than bFGF.

3.3. Effect of onapristone and mifepristone on serum stimulatory activity

We have already reported that mifepristone had inhibitory effects even at concentrations of 0.1 using 2.5 or 5% chFCS [10] and that it can also inhibit MPA-induced cell proliferation. The object of these experiments was to investigate the role of a different antiprogestin, which has a different mechanism of action [19]. Onapristone, at 1 nM concentration, inhibits cell proliferation ($P < 0.001$) and, at 10 nM concentration, it also inhibits cell proliferation induced with 10 nM MPA ($P < 0.001$) (Fig. 5).

3.4. Effect of mifepristone on bFGF stimulatory activity

To evaluate the possible involvement of PR in

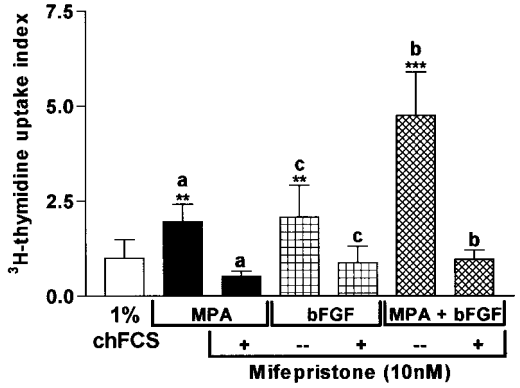

Fig. 6. Effect of mifepristone 10 nM on epithelial cell proliferation. The cells were grown in the presence of 1% chFCS and 10 nM MPA and/or bFGF 100 ng/ml for 48 h. The ^{3}H-thymidine index is the ratio between the total cpm counting (mean $\pm$ *SD*) of each experimental group ($n = 8$) and the control ($n = 16$, open bar). $^{**}P < 0.01$ vs control, $^{***}P < 0.001$ vs control, a and b: $P < 0.001$, c: $P < 0.01$. A representative experiment of other three is shown.

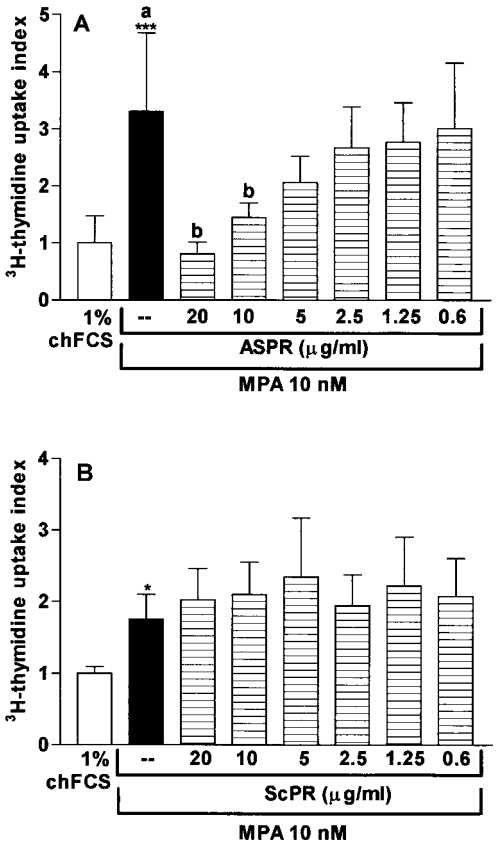

Fig. 7. Effect of ASPR (A) or ScPR (B) on ^{3}H-thymidine uptake in epithelial cells growing in the presence of 1% chFCS and 10 nM MPA incubated for 48 h. The ^{3}H-thymidine index is the ratio between the total cpm counting (mean $\pm$ *SD*) of each experimental group ($n = 8$) and the control ($n = 16$, open bar). $^{***}P < 0.001$ vs control, $^{*}P < 0.05$ vs control, a vs b: $P < 0.001$. A representative experiment of other three is shown.

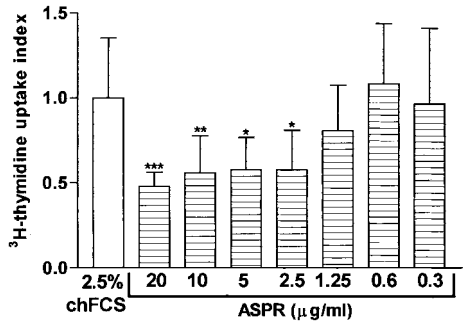

Fig. 8. Effect of ASPR on ^{3}H-thymidine uptake of epithelial cells growing in the presence of 2.5% chFCS incubated for 48 h. The ^{3}H-thymidine index is the ratio between the total cpm counting of each experimental group ($n = 8$) and the control ($n = 16$, open bar). $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ vs control. A representative experiment of other three is shown.

bFGF stimulatory activity we evaluated the ability of mifepristone to revert bFGF-induced stimulation. Mifepristone 10 nM inhibited bFGF (100 ng/ml) and MPA (10 nM) + bFGF-induced cell proliferation ($P < 0.01$). No statistically significant differences were found between MPA + mifepristone or bFGF + mifepristone groups (Fig. 6).

3.5. Effect of antisense PR on MPA, serum or bFGF induced cell proliferation

To further confirm the role of PR involvement in cell growth we investigated the effect of ASPR, which inhibited the proliferative effect of MPA in a dose-dependent manner (Fig. 7A); this inhibition was statistically significant at ASPR concentrations higher than 5 μg/ml. In the same range of concentrations ScPR had no effect (Fig. 7B). Similar experiments were performed using serum (Fig. 8) or bFGF (100 ng/ml)-

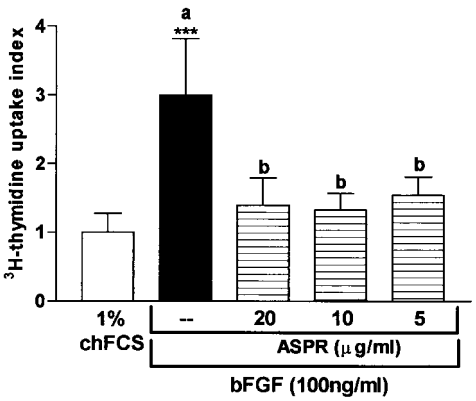

Fig. 9. Effect of ASPR on ^{3}H-thymidine uptake of epithelial cells growing in the presence of 1% chFCS and 100 ng/ml of bFGF incubated for 48 h. The ^{3}H-thymidine index is the ratio between the total cpm (mean ± SD) counting of each experimental group ($n = 8$) and the control ($n = 16$). $^{***}P < 0.001$ vs control, a vs b: $P < 0.001$. A representative experiment of other three is shown.

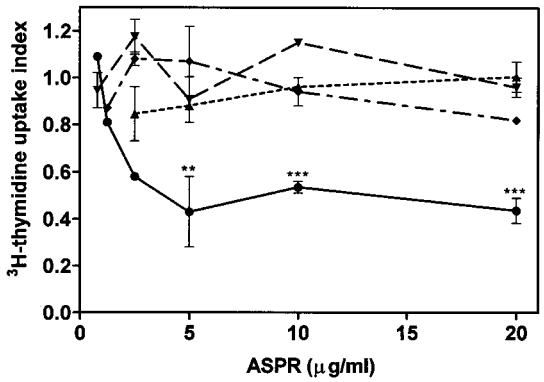

Fig. 10. Effect of ASPR on ^{3}H-thymidine uptake of C4-HD epithelial cells (●), stromal cells (▼), MCF-7 cells (▲) and NMuMG cells (◆) growing in the presence of 2.5–5% chFCS. The ^{3}H-thymidine index shown represents the mean value of 2–4 experiments. The ^{3}H-thymidine index in each experiment is the ratio between the total cpm counting of each experimental group ($n = 8$) and the control ($n = 16$). Values were statistically significant only with C4-HD cells. ($^{***}P < 0.001$, $^{**}P < 0.01$).

stimulated cells (Fig. 9). In both cases the inhibition was observed with ASPR concentrations higher than 2.5 μg/ml. The specificity of ASPR inhibition was also tested using primary cultures of fibroblastic stromal cells and also with two epithelial cell lines: MCF-7 cells and NMuMG cells. The results obtained with MCF-7 cells were similar to those reported in a previous paper [20]. A significant inhibition was observed only using C4-HD cells (Fig. 10). Using binding techniques, a significant decrease (59.3 ± 14%, $n = 3$) in ^{3}-H-R5020 binding was registered when cells were incubated in the presence of 20 μg/ml ASPR.

4. Discussion

The results reported herein demonstrate that the mitogenic activity induced by MPA depends on the presence of serum factors and that this mitogenic effect may also be induced with either aFGF or bFGF. These effects are mediated by the PR pathway since they can be reverted by two different antiprogestins and ASPR, suggesting a cross talk between different proliferative mechanisms.

The fact that serum factors are involved in steroid-induced cell proliferation has been documented for more than 20 years. Page et al. [21] demonstrated that estrogen sensitivity was lost in MCF-7 cells and that it could be restored by high concentrations of fetal calf serum. Soto and Sonnenschein [4] suggested that estrogens do not stimulate cell growth through ER, but that they rather induce cell proliferation by binding to plasma-borne specific inhibitors. They showed that an effect similar to that of E_2 can be achieved by serum dilution. A plasma derived serum inhibitor was

described by Dell'Áquila et al. [22] whose activity may be partially inhibited by heat inactivation. More recently Cho et al. [23] reported that the expression of PR can be modulated by E_2, cAMP, or IGF I, and that the addition of unknown serum factors may modify this response.

In our experiments charcoalization and heat inactivation seems to unmask certain serum factors capable of exerting a stimulatory effect which is specific for epithelial cells; this serum can stimulate thymidine uptake to levels similar to those obtained with MPA. These or other serum factors are also involved in the ability of progestins to stimulate cell growth, which is also enhanced when either one or both treatments are performed. Charcoalization eliminates all low molecular weight molecules including steroids, while heat inactivation is normally performed to inactivate complement. In our experiments using an autologous serum, it seems unlikely that the complement is playing a role, although terminal complement proteins, such as C5b-9 have been implied in the activation of pores responsible for the cellular release of some proteins such as FGFs [24]. The serum obtained from OVX animals treated with MPA exerted a stimulatory effect greater than that induced by the serum from OVX animals to which MPA was 'exogenously' added. This suggests that systemically administered MPA may be altering '*the growth factor milieu*'. Although we have not measured MPA levels in one week MPA-treated animals, we have previously reported levels around 10^{-7} M in MPA-treated animals which developed mammary tumors [7]. Since in previous experiments we showed similar stimulatory effects with MPA concentrations ranging 1 nM up to 100 nM, using either FCS or OVX serum [10], we decided to carry out these experiments using 10 nM concentrations of MPA.

Taken together, our results suggests that a progestin alone may be not sufficient to exert a proliferative effect, and that in order to have a full stimulatory effect, PR have to be activated by either a progestin in the presence of appropriate cofactors, or by a combination of factors. It can also be suggested from a different perspective, that serum manipulation blocks the action of inhibitors in such a way that the final balance favors the effect of growth factors. These factors would then stimulate cell growth by mechanisms which may bypass the PR pathway. In fact, serum factor-dependent signaling pathways may cross talk with PR, which is suggested by the fact that we were also able to demonstrate that an inhibition in serum and FGF-induced cell proliferation is obtained blocking the PR. This kind of ligand independent activation of steroid hormone receptors has also been postulated by others to explain the acquisition of the multihormone resistant phenotype [25].

The stimulation induced by FGFs suggest that these factors could be acting as progestomedins. Keratinocyte growth factor or FGF-7 is a progesterone-induced growth factor, synthesized by stromal cells, that might function as a progestomedin for epithelial cells in primate endometrium [26] and in mammary gland [27]. Although it can be hypothesized that FGFs could be acting as MPA-induced autocrine growth factors for epithelial cells, the fact that the proliferative effect is abolished by blocking PR, militates against a possible autocrine or paracrine role in hormone-dependent growth. It may be, however, possible that cells acquire an apparent hormone-independent growth by using the FGF pathway, which may in turn be using the same transcription factors, namely the PR pathway. Briozzo et al. [14] demonstrated that an increase in cathepsin D secretion may result in an increase in FGF availability. A similar mechanism may result in a progestin independent growth in our experimental model.

We used two antiprogestins with different mechanisms of action. Onapristone, a type I antiprogestin, does not induce a stable DNA binding of PR in vitro, suggesting that it inhibits the receptor activity at a step before DNA binding. Type II antiprogestins, including mifepristone, induce stable binding of PRs to DNA in vitro and in vivo but the resulting conformation is unable to induce transcription of progesterone-inducible genes [2,19,28]. Mifepristone consistently fails to induce the interaction of the PR ligand-binding domain with coactivators such as the steroid receptor co-activator I (SRC-1) and the transcriptional intermediary factor-2 (TIF2), which have been shown to play an important role mediating the effects of progestins [28,29]. It has recently been suggested that the complex mifepristone-PR_B may activate the transcription of genes without binding to the progesterone responsive elements [2,28]. Although, it may be speculated that mifepristone may inhibit serum or bFGF-induced proliferation bypassing the PR pathway, in our model, the fact that onapristone also inhibits serum-induced cell proliferation points towards a mechanism involving the PR. Steroid receptors can be modulated by different pathways, i.e. (1) the enhancement of the transactivation potential of dexamethasone-induced glucocorticoid receptor by 12-tetradecanoylphorbol 13-acetate, an inducer of the protein kinase C pathway [30], (2) the potentiation of agonist loaded steroid receptors with the protein kinase A pathway [31], (3) activation of ER through the MAP kinase pathway [32]. AFGF and bFGF are supposed to exert their proliferative effects using the MAP kinase pathway [33], and to our knowledge ours is the first description of a possible cross-talk between this pathway and PR.

It has recently been reported that FGF-transfected MCF-7 cells acquire an estrogen-independent growth

behavior and become resistant to several antiestrogens [34]. The authors suggested that this change may be achieved exclusively through the FGF signaling pathway, bypassing the steroid receptor mechanism. In our system the evidence points to FGF stimulating through the PR pathway. It can be speculated that the intracrine role of FGF may essentially be different from that of the exogenously added FGF; as suggested by others, FGF may act directly in the nucleus [35].

Soto and Sonnenschein [4] have put forward a negative hypothesis for estrogen or androgen sensitive tissues. As opposed to this hypothesis, in our experimental setting, a significant increase in thymidine uptake is observed at higher FCS concentrations, so that MPA's stimulatory effect is better observed when 1% chFCS is used. We have demonstrated that FGFs increase thymidine uptake at concentrations in which neither EGF, [11] nor IGF I or IGF II [12] exert any stimulatory effect. Although we routinely use thymidine uptake to measure cell proliferation, we performed cell counting in selected experiments which correlated with the former parameter. Another difference is that, as demonstrated in previous reports, the maximal proliferative response of MPA (10 nM) is obtained with concentrations ranging the Kd value of the PR. According to the negative hypothesis this should coincide with the shut off effect.

In summary, taken together our results suggest that tumor growth in our model involves a complex network of interconnected signals in which PR play an important role. This role is essential enough for the PR to be used as a common pathway by different proliferative mechanisms, some of them unrelated to its specific ligands.

Acknowledgements

We thank Dr. M Schenider from Schering, Germany for gently providing the onapristone, Roussel Uclaf for the mifepristone and Laboratorios Gador, Buenos Aires for the medroxyprogesterone acetate. We also thank Dr. CD Pasqualini, Academia Nacional de Medicina, Buenos Aires, for kindly providing the BALB/c female. C.L. is fellow of FUNDACION SALES and C.L*. is member of Research Career (CONICET). This study was supported by grants of CONICET: PMT.PICT 0446 Préstamo BID 802/OC-AR, Fundación Sales, Fundación Roemmers and Laboratorios Schering Argentina.

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