Prolactin Binds to Human Endometrial Fibroblasts and Inhibits Mitogenicity of an Endometrial Carcinoma Extract (43581)

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Abstract. **Uterine endometrial carcinoma has been reported to synthesize and secrete a putative peptide mitogen that elicits a potent proliferative response in endometrial fibroblasts. The extract from endometrial carcinoma stimulated [^{3}H]thymidine incorporation into human endometrial fibroblasts in a dose-dependent manner. Concomitant exposure of the fibroblasts to prolactin (PRL) led to a remarkable inhibition of the extract-stimulated mitogenic activity of the fibroblasts. This inhibition was dependent on PRL dose, and maximal effect occurred at 1 μM of PRL. PRL (10 nM) suppressed an apparent maximal activity of the extract by 50%, and the half-maximal stimulated effect of the extract on thymidine incorporation was observed at the same concentration in the absence or presence of PRL. This noncompetitive manner may imply that PRL acts at a stage after the interaction of the mitogen in the extract with the specific receptor for mitogen. When the fibroblasts were first exposed to the extract for 12 hr and then to PRL, PRL suppressed the mitogenic activity with no lag. The rapid growth-inhibitory effect of PRL was mimicked by prostaglandin E_1, but the combination of both types of ligand was not additive in the inhibitory action on growth. PRL and prostaglandin E_1 may inhibit a similar mitogenic signaling cascade.**

Specific receptor sites for PRL were detected in the endometrial fibroblasts, showing high binding affinity (K_d = 16.1 nM) and low binding capacity (B_{max} = 1.59 pmol/mg protein). Treatment of the fibroblasts with the endometrial carcinoma extract induced no changes in the level of PRL receptor, excluding the possibility that PRL competes for the binding sites with the mitogen. These findings would suggest that PRL may block the mitogenic activity of the fibroblasts stimulated by the endometrial carcinoma-derived mitogen via a PRL receptor-mediated mechanism, perhaps prostaglandin production.

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It is well known that carcinoma cells and stromal cells such as fibroblasts coregulate their proliferation *in vivo* and *in vitro* (1–6). The presence of mitogenic and/or chemotactic activity has been documented in culture medium conditioned by various carcinoma cells (1–4), and in ascitic fluid or urine from patients with carcinomas (7–9). More recently, we reported that uterine cervical and endometrial carcinomas synthesize and secrete a putative peptide growth factor(s) for fibroblasts (10, 11). These growth factors may exert their effects by a paracrine or autocrine mechanism, i.e., they may be produced and released in the immediate vicinity of their sites of action.

Uterine endometrium and endometrial carcinoma respond to reproductive hormones, including sex steroids and prostaglandins (PG) (12–14). Recent data indicate an important role for prolactin (PRL) on human endometrium. PRL is synthesized and released from human endometrium during the late secretory phase and increased with decidualization (12), and exerts its biologic effects on endometrial cells (13). For the initiation of biologic action, peptide hormones and growth factors must first bind to specific membrane receptors on target cells (1, 4, 15–17). Therefore, in this study, the PRL receptor was characterized in human

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endometrial fibroblasts. As a first step in the detection of a possible control factor on the mitogen in the endometrial carcinoma extract, the effect of PRL on the growth of endometrial fibroblasts in response to endometrial carcinoma extracts was also examined and compared with that of the arachidonic acid metabolite PG.

Materials and Methods

Preparation of Human Endometrial Fibroblasts. Specimens of normal endometrium were obtained from patients undergoing elective hysterectomy for uterine liomyoma or dilatation and curettage as a screening test for carcinoma. The tissues were trimmed and washed with Hanks' balanced salt solution, and subsequently treated for 1.5 hr at 37°C with 0.25% collagenase (type I) in Hanks' balanced salt solution, as described previously (10, 11). The cell suspension was filtered through nylon mesh to remove tissue fragments, cell debris, and fibers. The filtrate was then centrifuged and washed with Hanks' balanced salt solution. The cells in the pellet were resuspended and incubated in modified minimum essential medium, Eagle's minimal essential medium supplemented with 10% fetal bovine serum. After several days of incubation, nonadherent cells were removed, and the fibroblasts were grown as a monolayer. The growth medium was changed every second day until confluence was reached, and these were maintained in the above medium as a stock culture. One stock culture was prepared from one patient, and the experiments were always performed using three stock cultures. No significant difference was detected between fibroblasts from secretory phase-endometrium and in the cells from proliferative phase-endometrium in our assay.

PRL Receptor Binding in Human Endometrial Fibroblasts. The binding of ^{125}I-human PRL to whole endometrial fibroblasts was performed as described previously (15). Briefly, 1×10^6 cells/ml were resuspended in balanced salt solution (135 m*M* NaCl, 4.5 m*M* KCl, 1.5 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 5.6 m*M* glucose, and 10 m*M* HEPES, pH 7.4) and incubated at 0–4°C with 20–200 fmol of tracer ^{125}I-PRL and increasing amounts of unlabeled PRL in a total volume of 0.5 ml. In additional tubes, a 500-fold excess of PRL was added at the beginning of the incubation to evaluate nonspecific binding. The binding experiments were performed in polypropylene tubes. After 12 hr of incubation, the cell suspensions were diluted with 10 vol of ice-cold balanced salt solution. The cells were then washed twice with ice-cold balanced salt solution to remove unincorporated ^{125}I-PRL. The cells were pelleted by centrifugation at 400*g* for 5 min, and radioactivity in the pellet was counted.

Preparation of Extract from Human Uterine Endometrial Carcinoma. Uterine endometrial carcinomas were placed in ice-cold phosphate-buffered saline immediately after surgical removal and representative portions were excised to prepare the material for histologic frozen sections. These tumor samples were washed, and immediately used or stored in liquid nitrogen. Specimens found histologically to represent well-differentiated adenocarcinoma were submitted to separate experiments. After washing at 0–4°C with phosphate-buffered saline, a volume of homogenizing buffer (0.25 *M* sucrose, 1 m*M* EGTA, and 10 m*M* HEPES, pH 7.4) was added equal to four to five times the volume of the removed tissue. They (~10 g) were subsequently homogenized at 0–4°C using a Teflon homogenizer. The homogenate was then centrifuged at 100,000*g* for 1 hr and the resulting supernatant ("extract") was stored in liquid nitrogen (10, 11). After determining the growth-promoting activity of individual samples as described below, the activity positive extracts were pooled, and five different pools were used in the present experiments. The minimum amount of activity required for pooling was 3.5×10^3 dpm of [^{3}H] thymidine incorporation in a standard assay condition; four of 15 specimens had less activity. The range of activities in the five pools was $4.6 \pm 0.55 \times 10^3$ dpm. Protein content was determined by the method of Lowry *et al.* (18) with bovine serum albumin as a standard.

[^{3}H]Thymidine Incorporation into Endometrial Fibroblasts. DNA synthesis was assayed by measuring the incorporation of [^{3}H]thymidine into the trichloroacetic acid-insoluble fraction (10, 11). Before experimentation, the confluent cells were seeded into plastic 24-well plates at a density of 1.0×10^5 cells/cm^2. At confluence, monolayer cultures were preincubated in mineral essential medium with 1% fetal bovine serum for 2 days, and submitted to concurrent exposures to various agents to be tested at 37°C. PRL was dissolved in sterile culture medium, and PG in dimethyl sulfoxide; the dimethyl sulfoxide concentration in cultures was less than 0.1%, which had no effect on fibroblast DNA synthesis. [^{3}H]Thymidine incorporation was examined by a continuous or a 2-hr pulse labeling with 1 μCi/well, and uptake was quenched by aspiration of media. The cells were washed with ice-cold 0.5% trichloroacetic acid and washed three times by centrifugation (400*g*, 5 min). The radioactivity associated with acid-insoluble material at the bottom of the tube was counted.

Statistics. Statistical analysis was performed by *t* tests. Differences were considered significant if $P < 0.01$.

Materials. [6-^{3}H]Thymidine (1.11 TBq/mmol), and ^{125}I-human prolactin (1.85 MBq/μg) were obtained from New England Nuclear. Prolactin (human, 30 IU/

mg) and prostaglandins were from Sigma, and all chemicals were of reagent grade.

Results

Effects of PRL on Mitogenic Activity of Human Endometrial Fibroblasts. Growth-promoting activity was examined by pulse labeling with radioactive thymidine of DNA in human endometrial fibroblasts. The time course of thymidine incorporation in the presence or absence of extract is shown in Figure 1. Maximal stimulation in the presence of extract occurred after 24 hr of incubation, whereas basal mitogenic activity (in the absence of extract) was constant up to 32 hr in these conditions. Concomitant exposure of the fibroblasts to PRL (1 μM) led to a remarkable inhibition of the extract-stimulated thymidine incorporation; PRL alone had no significant effects on thymidine incorporation (Fig. 1). This concentration of PRL seemed to suppress the stimulated rate of thymidine uptake to the control level. The inhibitory action of PRL was dose dependent, and the maximal effect occurred at 1 μM (Fig. 2). The half-maximal inhibitory effect of PRL occurred at a concentration between 1 and 10 nM. PRL alone did not stimulate [^{3}H]thymidine incorporation into the fibroblasts.

Figure 3 illustrates the dose dependency of thymidine incorporation by the extract in the presence or absence of PRL. Exposure of the fibroblasts to the extract increased the rate of thymidine uptake in a dose-dependent manner; maximal activity was obtained at 10 μg of protein. In the presence of 10 nM

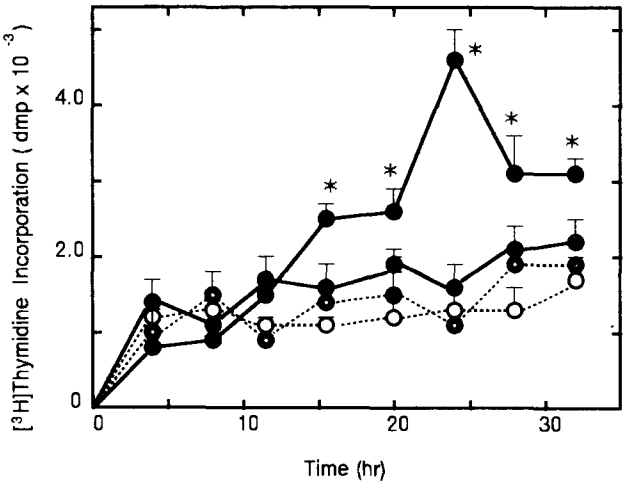

Figure 1. Effect of PRL on mitogenic activity of human endometrial fibroblasts in response to extract from uterine endometrial carcinoma. Cultured endometrial fibroblasts (2 × 10^5 cells/ml) were incubated for the indicated time intervals with extract from endometrial carcinoma (20 μg of protein) (●), PRL (1.0 μM) (◎), extract from endometrial carcinoma plus PRL (◉), or vehicle (○). Mitogenic activity was measured by a 2-hr pulse labeling with [^{3}H]thymidine (1 μCi). The experiments were performed using three stock fibroblast cultures, and each point represents the mean ± SD of the determinations. *$P < 0.01$ versus control.

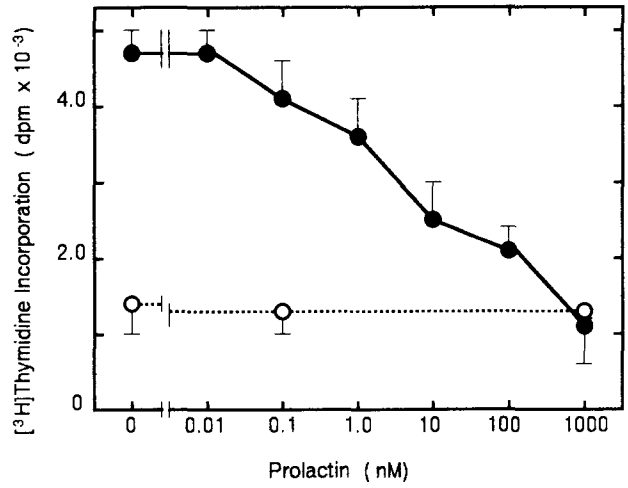

Figure 2. Effect of increasing doses of PRL on mitogenic activity of human endometrial fibroblasts in response to extract from uterine endometrial carcinoma. Cultured endometrial fibroblasts (2 × 10^5 cells/ml) were incubated for 24 hr with various concentrations of PRL in the presence of extract from endometrial carcinoma (20 μg of protein) (●) or vehicle (○). Mitogenic activity was measured by a 2-hr pulse labeling with [^{3}H]thymidine (1 μCi). The experiments were performed using three stock fibroblast cultures and two different pools of extract. Each point represents the mean ± SD of the determinations.

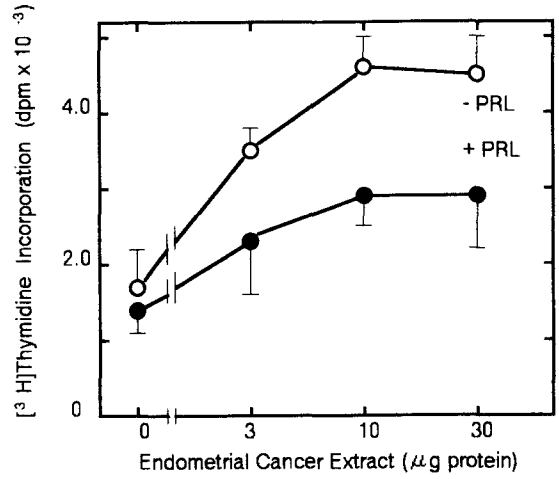

Figure 3. Concentration dependence of endometrial carcinoma extract-stimulated mitogenic activity in control endometrial fibroblasts and in cells exposed to PRL. Cultured endometrial fibroblasts (2 × 10^5 cells/ml) were incubated for 24 hr with various concentrations of extract from uterine endometrial carcinoma, in the presence (●) or absence of (○) PRL (10 nM). Mitogenic activity was measured by a 2-hr pulse labeling with [^{3}H]thymidine (1 μCi). The experiments were performed using three stock fibroblast cultures and two different pools of extract. Each point represents the mean ± SD of the determinations.

PRL, the maximal thymidine uptake in response to the extract was suppressed by approximately 50% of the extract-stimulated level. A higher concentration of the extract did not overcome the inhibitory effects of PRL on growth-promoting activity. A half-maximal effect on thymidine incorporation was observed at the same concentration (approximately 3 μg of protein) in the

absence and presence of PRL, suggesting that PRL inhibited the mitogenicity of the extract in a noncompetitive manner.

The effects of PRL on extract-induced growth was then evaluated in an experiment in which the cells were first activated by the extract. As shown in Figure 4, after 3 hr or 12 hr of incubation with the extract, the addition of PRL (1 μM) reduced the rate of mitogenic activity to the control rate. PRL could suppress mitogenesis when it was added up to 15 hr after addition of the extract.

Effects of Prostaglandins on Thymidine Incorporation in Human Endometrial Fibroblasts. The effects of prostaglandin E_1 on the growth-promoting activity of the extract were examined by a continuous labeling with radioactive thymidine (Fig. 5). PGE_1 (1 μM) had inhibitory effects on the growth-promoting activity in an analogous manner to PRL. In the presence of the prostanoid, there was a net decrease in thymidine incorporation due to endometrial carcinoma extract of approximately 60% of that induced by the extract. Basal thymidine uptake activity was not significantly influenced by the prostanoid. $PGF_{2\alpha}$ showed similar effects, but PGE_2 had a lesser effect (data not shown). The combination of PGE_1 (1 μM) and PRL (10 nM) had no greater effect than the effect of PRL (1 μM) alone. The simultaneous stimulation by these two did not change the maximal effects of the extract.

Specific Binding Sites for PRL in Human Endometrial Fibroblasts. Specific binding sites for PRL, in significant amount and affinity, were found in the endometrial fibroblasts. The characteristics of binding are shown in Figure 6. The nonspecific binding was high but relatively constant. The specific binding was reproducible and only one class of binding sites was found. The affinity of binding was in the nanomolar range (K_d = 16.1 nM), and the capacity of receptors was 1.63 pmol/mg of protein. The simultaneous presence of the endometrial carcinoma extract affected neither the PRL receptor concentration nor its affinity for PRL.

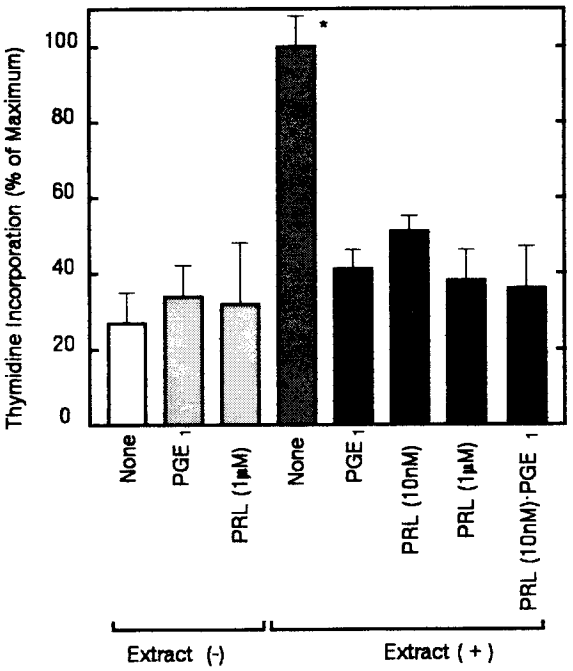

Figure 5. Effects of PGE_1 or PRL on endometrial carcinoma extract-stimulated mitogenic activity in endometrial fibroblasts. Cultured endometrial fibroblasts (2 × 10^5 cells/ml) were incubated for 16 hr with either PGE_1 (10 nM), PRL (10 nM, or 1 μM), or both in the presence or absence of endometrial carcinoma extract (20 μg of protein). Mitogenic activity was measured by continuous labeling with [^{3}H] thymidine (0.5 μCi), and the data are expressed as percentage of thymidine incorporation in extract-stimulated cells. *$P < 0.005$ versus control.

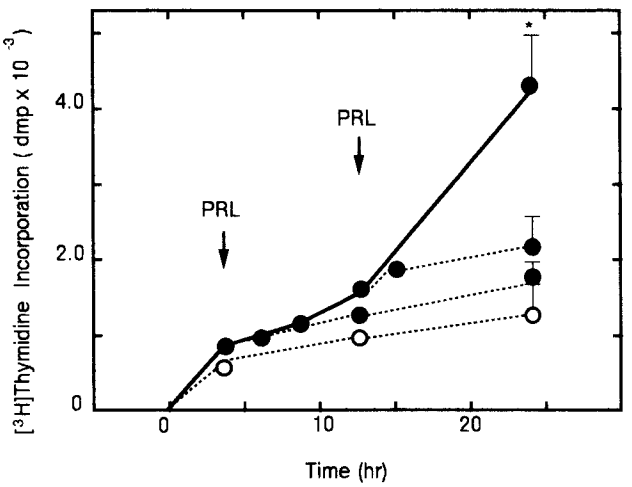

Figure 4. Time course of PRL effect on endometrial carcinoma extract-stimulated mitogenic activity in endometrial fibroblasts. Cultured endometrial fibroblasts (2 × 10^5 cells/ml) were incubated for indicated time intervals with (●) or without (○) carcinoma extract (20 μg of protein). At time intervals indicated by an arrow, PRL (1 μM) was added to portions of the cell suspension previously exposed to the corpus carcinoma extract (◉). Mitogenic activity was measured by a 2-hr pulse labeling with [^{3}H]thymidine (1 μCi). The experiments were performed using three stock fibroblast cultures and two different pools of extract. Each point represents the mean ± SD of the determinations. *$P < 0.001$ versus PRL-added portions.

Discussion

Extract from endometrial carcinomas, but not from benign tumor myoma or normal tissue, contains a putative growth-promoting peptide(s) for endometrial fibroblasts (10, 11, 19). The endometrium has been demonstrated to have specific receptors for certain reproductive hormones, suggesting that these hormones may play a role in the growth of the endometrium and its derived tumors, such as endometrial cancer. The results presented here showed the presence of specific binding sites for PRL in the endometrial fibroblasts. This finding led to the idea that PRL may interfere with the effect of carcinoma extract on endometrial fibroblast growth. It was observed that PRL caused a marked

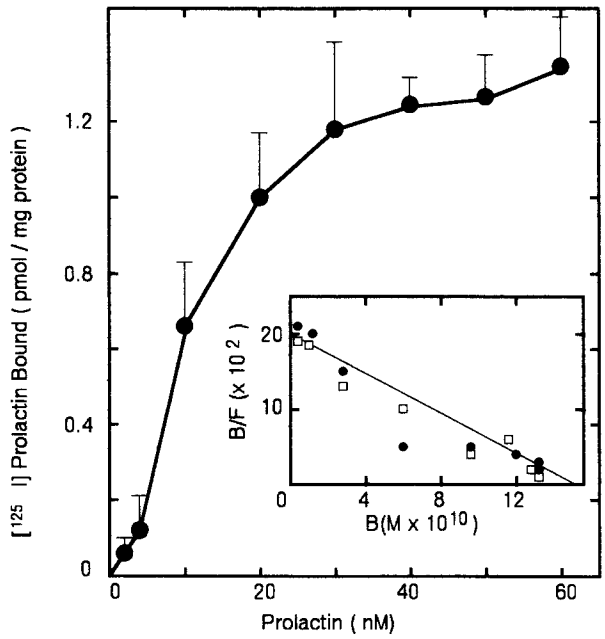

Figure 6. Specific binding of [^{125}I]-PRL to human endometrial fibroblasts as a function of PRL concentration. Cultured endometrial fibroblasts (2×10^5 cells/ml) were incubated for 12 hr with various concentrations of [^{125}I]-PRL at 0–4°C. Specifically bound PRL was calculated as the amount of radioactivity not displaced by the addition of a 500-fold excess of unlabeled PRL. Each point represents the mean ± SD of triplicate determinations of two separate experiments. The inset shows a Scatchard plot of the data in the absence (●) or presence of endometrial carcinoma extract (□) from which an equilibrium dissociation constant (K_d) of 17.1 n*M* and maximal binding capacity (B_{max}) of 1.59 pmol/mg of protein were calculated.

inhibition on mitogenicity of an endometrial carcinoma extract.

The first step in the interaction of many peptides and growth factors with the fibroblasts is binding of the factors to specific receptors (1, 4, 15, 16). The binding of the factors promotes the generation of early signals in the membrane and cytosol, including stimulation of phosphoinositide metabolism and protein kinase C (1, 4, 15, 16). Although the mechanism by which fibroblasts respond to the putative growth factor in the endometrial carcinoma extract is not clear, the activation of protein kinase C is considered as a possible link in the chain of events that synergistically enhances the growth-promoting activity (10). Kalbag *et al.* (13) have demonstrated the presence of protein kinase C in human endometrium and suggested that human PRL stimulates protein kinase C activity in the endometrium. The protein kinase C in the endometrial fibroblasts must be stimulated by PRL receptor occupancy. The chronic stimulation by PRL may induce downregulation of protein kinase C in the fibroblast with a consequent decline of mitogenic activity. The downregulation of protein kinase C seems to be involved in the growth-inhibiting activity of PRL on the fibroblasts in response to endometrial carcinoma extracts.

The receptor-associated membrane events rapidly follow the receptor occupancy. The translocation and activation of protein kinase C in endometrial cells are reported to occur within 20 min after exposure to PRL (13). Long-term incubation for over 2 hr results in a loss in protein kinase C activity. In our experiments, the time required for PRL-induced growth inhibitory events was on the order of hours, and after the growth-promoting activity was started, PRL had an inhibitory action on growth with a lag of several hours. It appears to be reasonable that the effect of PRL results from the downregulation of protein kinase C.

The involvement of a protein kinase C-dependent mechanism, intracellular event, in PRL action may agree with two data presented in this paper. (i) We detected a high affinity receptor site for PRL in the human endometrial fibroblasts similar to other PRL target tissues (20–23). The endometrial carcinoma extract affected neither PRL receptor concentration nor its affinity for PRL. This may exclude the possibility that PRL blocks the growth-promoting activity through the displacement of previously bound mitogen in the extract from its binding site. (ii) PRL inhibits the mitogenicity of the extract in a noncompetitive manner, which may imply that PRL acts at a locus after the interaction of growth-promoting peptide and its receptor.

Assuming then, that PRL initially does interact with specific receptor sites on the cell surface, one may then inquire as to the mechanism by which intracellular metabolic processes are altered in response to PRL. Since the actions of other peptide hormones, including relatively large polypeptides, have been shown to be carried out by intracellular mediators, it is indeed possible that the PRL effects occur by one or more of these messengers (15, 16). Evidence for an alternative interpretation has been presented that suggests that PRL may be internalized into its target cells as a form of PRL-receptor complex and have intracellular actions to generate intracellular mediators (24, 25). Among proposed intracellular mediators of PRL action, the most likely candidate may be a stimulation of phospholipase A_2 activity and a subsequently enhanced synthetic rate of a specific prostaglandin in the target cells (24, 25). This concept is in close agreement with our findings. The action of PRL on endometrial fibroblasts was mimicked by arachidonic acid metabolites, in particular PGE_1. The combination of PGE_1 and PRL had no greater effects than the effect of PRL alone, suggesting that PRL and PGE_1 share a similar acting cascade in the fibroblasts. However, the relationship between PG and protein kinase C is unclear in the endometrium.

The putative mitogen in the endometrial carcinoma extract could be produced and released in the immediate vicinity of its site of action. The function of the mitogen for endometrial fibroblasts can most rea-

sonably be assumed to be that of local hormone-like agents that can reversibly control cell function by a paracrine mechanism. Endometrial carcinoma cells require co-growth of supporting cells, such as stromal cells, for their own growth. Since PRL is synthesized by endometrial stroma (12) and binds to endometrial fibroblasts and possess antigrowth activity against the mitogenic activity from the carcinoma, this new action of PRL can give a better insight as to the mechanism of carcinoma growth and invasion, and facilitate the prediction of responses to hormonal therapy.

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