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Corticotropin Releasing Factor Decidualizes Human Endometrial Stromal Cells *In Vitro*. Interaction with Progestin

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Decidualization of endometrial cells is a hormone-dependent process of differentiation which occurs during the menstrual cycle and pregnancy. Recent *in vitro* studies have revealed that cAMP and its generators induce decidualization of stromal cells isolated from proliferative endometrium and that progestins enhance the effect of cAMP. Since corticotropin releasing factor (CRF) generates cAMP and prostaglandins in other organs, in the present study the effect of CRF, a hypothalamic factor also produced by decidua and fetal membranes, on *in vitro* decidualization of endometrial stromal cells was evaluated. The addition of CRF to a culture medium of stromal cells induced *in vitro* decidualization, as indicated by morphologic changes from elongated fibroblast-like cells into larger and round cells and by the release of prolactin in the medium. The effect of CRF on stromal cells and on prolactin release was significantly augmented by the coincubation in the presence of medroxyprogesterone acetate. This observation indicates CRF as a novel factor of decidualization and confirms that progestins act as enhancers of the expression of decidual products.

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INTRODUCTION

Decidualization of human endometrial stromal cells, occurring during the secretory phase of the menstrual cycle and during pregnancy, involves extensive cell differentiation characterized by the production of hypophyseal hormones (prolactin, oxytocin), cytokines (colony stimulating factor-1, tumor necrosis factor, interleukin 1 and interleukin 6), extracellular matrix components (laminin, fibronectin, collagen IV, heparan sulfate proteoglican), neuropeptides (somatostatin, inhibin, activin, corticotropin releasing factor), as well as a variety of enzymes (diamine oxidase, aromatase, steroid sulfatase, proteases, protease inhibitors) [1].

The decidualization process is considered to be physiologically induced by progesterone, the typical steroid hormone secreted during the luteal phase of the menstrual cycle and during pregnancy. However, recent *in vitro* findings revealed that dibutyryl cAMP or other compounds generating cAMP, such as gonadotropins (FSH, LH, CG), are primary inducers of differentiation of endometrial stromal cells [2–4]. The role that progestins play in decidualization may result from their capability to drastically enhance the effect of cAMP and its generators on the differentiation process.

In addition to being a hypophysiotropic neurohormone, CRF is also shown to be produced by decidual cells by either measuring mRNA levels or by the detection of immunoreactive CRF in cells as well as in culture medium [5, 6]. In placenta and fetal membranes, as well as in decidua, CRF has been shown to induce ACTH [7], PGF_2 and PGE_2 [8, 9] release and it is a generator of cAMP [7]. CRF is a potent stimulator of the cAMP pathway in all target cells [10, 11]. In the present study, we tested the effect of CRF as an *in vitro* decidualizing substance, in the presence or in absence of medroxyprogesterone acetate (MPA). Differentiation of stromal to decidual cells was followed by using morphologic and biochemical markers.

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MATERIALS AND METHODS

Isolation of stromal cells from human endometrium

Endometrial tissue specimens were excised from uteri of normally cycling women undergoing hysterectomy for leyomyomas. Only normal proliferative endometrium, histologically dated according to the criteria of Noyes *et al.* [12], were used for the experiment described here. After thorough cleaning and trimming to remove blood clots and mucus, the specimens were minced to fragments of less than 1 mm^3 size under a laminar flow hood and then digested at 37°C for 90 min with 0.25% collagenase (Worthington Biochem. Co, Freehold, NJ) in Ham's-F10 medium containing 10% charcoal-treated fetal bovine serum (ct-FBS). As previously described [13], the suspension was passed through a 25 μm pore size stainless steel sieve in order to separate stromal cells from glands. The collected cells were resuspended in RPMI 1640 medium with 10% ct-FBS containing 0.2 U/ml insulin (Squibb & Sons Inc., Princeton, NJ) and 1% antibiotic–antimycotic solution (GIBCO), plated for 30 min in plastic culture dishes and incubated in a 95% air–5% CO_2 atmosphere at 37 C. Unattached cells were discarded and the remaining morphologically homogeneous population of attached stromal cells was allowed to reach confluency.

Culture of endometrial stromal cells with corticotropin releasing factor (CRF)

Endometrial stromal cells, forming a confluent monolayer in 3 cm dishes, were cultured in RPMI 1640, 0.1U/ml insulin, 2% stripped calf bovine serum (ct-CBS), 1% antibiotic-antimycotic solution, 0.2 mM of 3-isobutyl-1-methylxanthine (IBMX) to serve as controls. The same medium with CRF added at various concentrations served as the test group. Medroxyprogesterone acetate (MPA) was included in the media containing CRF in order to test for the influence of progestins on the expression of CRF effects. Furthermore, cultures with either IBMX alone, E_2 and MPA, or dybutyryl-cAMP were tested to evaluate their influence on morphology and on the induction of decidual products in the stromal cells.

In a separate experiment, helical CRF (9-41), a competitive synthetic antagonist of CRF [14], was added at the same concentration as CRF.

Media containing CRF or db-cAMP was changed every 24 h while media containing E_2 and MPA were changed every 2 days. All cultures were carried out in duplicate.

Morphology

Morphologic changes of cells cultured under different experimental conditions were examined daily with an inverted microscope and by phase contrast microscopy.

Immunocytochemistry

Endometrial stromal cells were tested for prolactin (PRL), desmin, fibronectin and calcitonin by using a streptavidin/biotin method. Cell cultures on Lab-Tek slides (Nunc Inc., Naperville, IL) under different experimental conditions were washed with cold hydrosaline solution and fixed for 5 min in 3% formalin. After blocking of non-specific reactivity, slides were covered by the primary antibody (Immunon, Pittsburg, PA, U.S.A.) for 18 h at 4°C. Slides were then incubated for 30 min at room temperature with the biotinylated secondary antibody. In the following step slides were incubated with streptavidin-peroxidase reagent and washed after 30 min. The 3-amino-9-ethycarbazole chromogen solution was then added for the development of color. A further counterstaining with hematoxilin followed before mounting cover slides with gel.

PRL assay

In order to evaluate the effect of CRF on the production of PRL by stromal cells, samples of cultures with or without CRF were collected every 24 h and centrifuged at 1000 ***g*** to separate floating cells and debris. The supernatants were frozen and kept at −20 C until assayed. Levels of prolactin in the medium were measured by performing solid-phase sandwich-type enzyme-linked immunosorbent assay (ELISA), using kits purchased from Leinco Technologies, Inc., St Louis, MO, in which wells in plastic plates were coated with anti-PRL murine monoclonal antibody to bind added PRL in samples or controls. Addition of anti-PRL goat polyclonal antibody labeled with horseradish peroxidase allowed

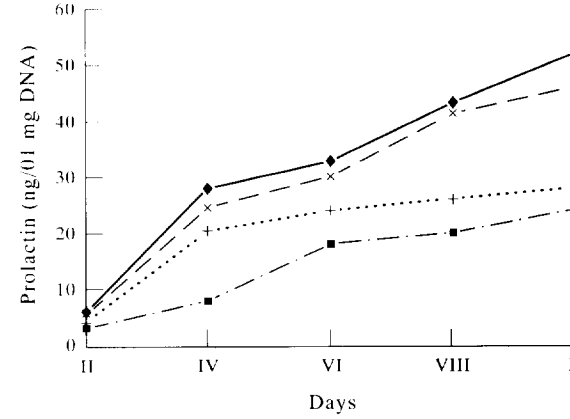

Fig. 1. Time-dependence and dose–response of CRF-induced PRL output from stromal cells isolated from human proliferative endometrium. Confluent stromal cells were cultured in RPMI-1640 and 2% ct-CBS for 10 days with CRF at 35 (■), 150 (+), 250 (×) and 350 (◆) nM. Prolactin levels were determined by RIA in medium collected every 24 h. The values represent the mean of duplicate determinations from three separate cultures. Levels of PRL in control samples were not measurable (less than 2 ng/ml). No temporal differences in the number of cells for each culture were observed.

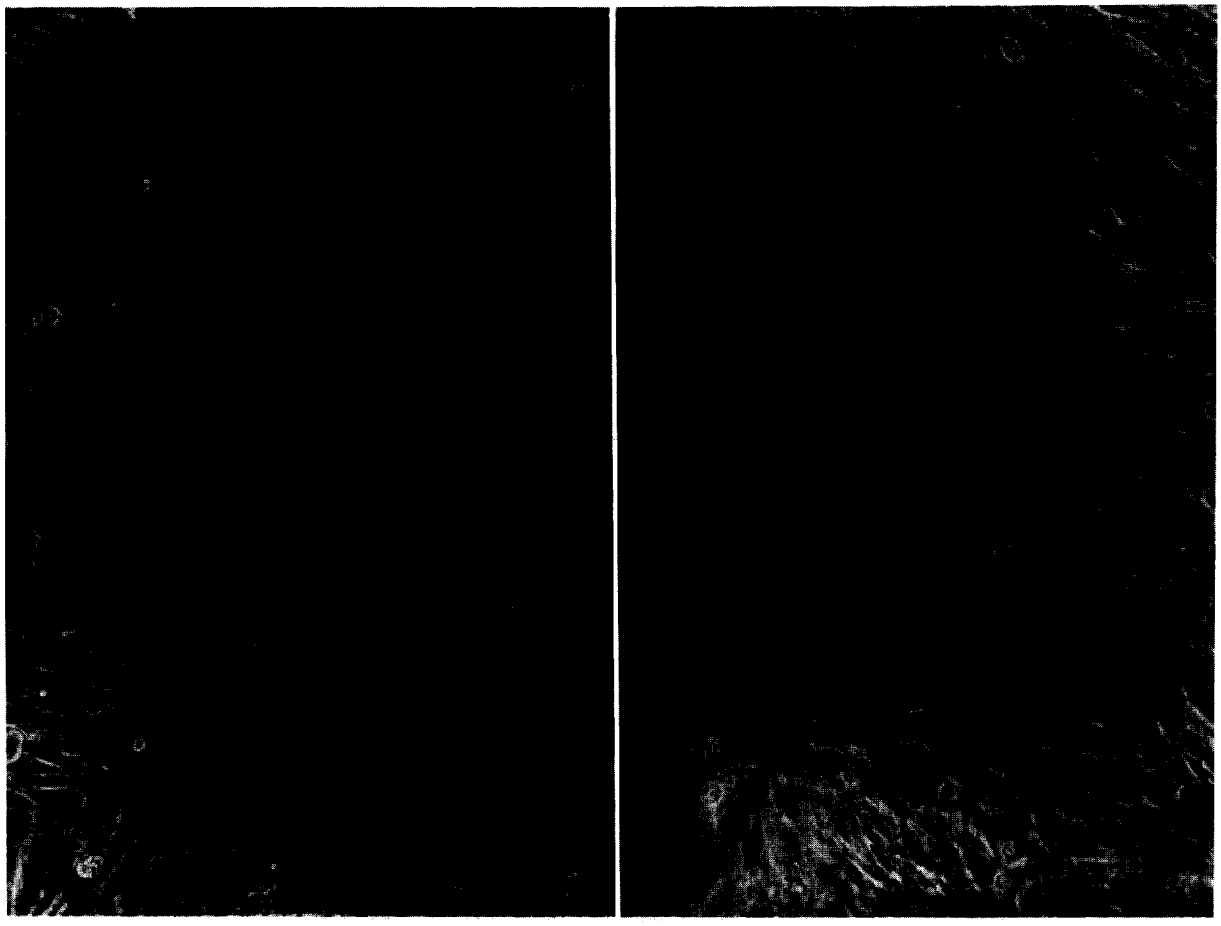

Fig. 2. Morphological appearance of stromal cells isolated from human proliferative endometrium cultured in RPMI/ct-CBS without (A) or with 150 nM CRF (B) for 8 days and then photographed under contrast microscopy (× 100)

colorimetric evaluation of PRL with a substrate chromogen.

RESULTS

In cultured stromal cells isolated from proliferative endometrium, CRF increases the release of PRL, a characteristic decidual product (Fig. 1). The effect of CRF was time- (from 2 to 10 days) ($P < 0.01$) and dose-dependent (from 35 to 350 nM) ($P < 0.01$). The expression of other markers of decidualization, such as desmin, fibronectin and calcitonin was also demonstrated immunocytochemically in cells treated with CRF (data not shown). These biochemical changes were associated with morphologic aspects from spindle-shaped stromal cells into larger round cells of the decidual phenotype (Fig. 2), thus further demonstrating the decidualizing effects of CRF.

Figure 3 indicates that the CRF-induced decidualization is inhibited by helical CRF (9-41), probably by competition for the CRF receptor. Medroxyprogesterone acetate enhances the effect of CRF,

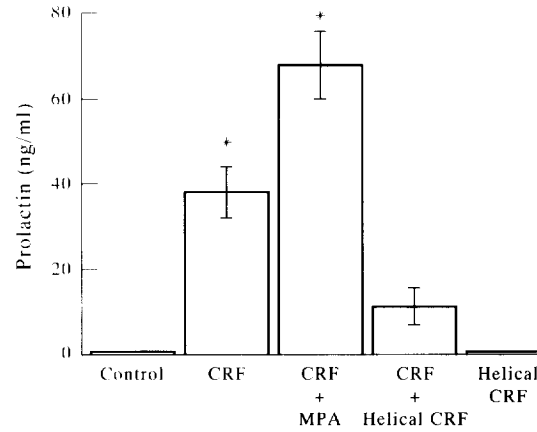

Fig. 3. Effects of CRF, CRF plus MPA, CRF plus helical CRF (9-41) and helical CRF (9-41) on PRL output from human endometrial stromal cells cultured for 8 days. Media collected during the last 24 h were measured for prolactin determined by RIA. The concentrations of PRL in cultures of cells untreated with CRF or treated with helical CRF were not measurable (less than 2 ng/ml). The mean and SD for various treatments are reported. Student's *t*-test was applied for determination of statistical significance of differences in the presence or absence of CRF (*$P < 0.01$).

doubling the output of PRL ($P < 0.01$). No significant changes of PRL release were observed when MPA was added in absence of CRF (data not shown).

DISCUSSION

The present findings allow the inclusion of CRF in the group of bioactive compounds exerting decidualizing effects on cultured stromal endometrial stromal cells. In addition a synergistic interaction of CRF with MPA is also demonstrated. Considering the capability of CRF to generate cAMP 10, the present observation confirms that factors activating decidualization have the common characteristic to generate cAMP (hCG, FSH, LH and prostaglandin E_2) [2, 4]. CRF receptors on endometrial stromal cells have not yet been described, however recent observation indicates a number of CRF receptors in human myometrial cells [15]. Again, in this tissue CRF receptors are coupled to the cAMP pathway [15].

The hypothesis that cAMP modulates the decidualization action of CRF, is based upon the demonstration that CRF acts throughout cAMP in the various target organs (brain, pituitary, spleen, placenta) [10, 11] with the only exception being the testis (Leydig cells) where CRF receptors activate the protein kinase C pathway [16]. Similarly, the enhancement by MPA of the effect of CRF on decidual PRL production is consistent with the previous reports showing a potentiation of MPA on the decidualizing effect of cAMP [3].

The question which remains to be elucidated is the source of the CRF acting on endometrial cells. Endometrial stromal cells in culture express immunoreactive CRF following incubation with steroid hormones [9]. Since this experimental procedure is associated with decidualization *in vitro*, it is possible to hypothesize that the process of decidualization involves CRF production and action. However, it is not possible to exclude that CRF acting on endometrium may derive from immune cells colonizing the endometrium or from systemic circulation. In fact, immune competent cells (leukocytes, macrophages) synthesize and produce bioactive CRF [17–19].

The physiologic and clinical significances of this new type of decidualizing agent remains to be explored. The process of decidualization is fundamental towards embryo implantation and gestational development. A modulatory role of CRF on lymphocyte chemiotaxis and -interferon production may support an action on local cell mediated immune reaction [19, 20]. Similarly, the increased expression of CRF mRNA in decidual cells during healthy gestation supports a functional role in the paracrine regulation of decidual and fetal membrane interaction [5]. A relevant role for CRF has been suggested in regulating prostaglandin release from decidua around the time of delivery; a putative biochemical key event in the mechanisms of parturition [8, 9, 21]. Moreover, the present data suggest that decidual CRF may also participate in the regulation of local prolactin release during the course of gestation. Since PRL is considered one of the major marker/effectors of the decidual process, the effectiveness of CRF in increasing decidual PRL release suggests a possible role of CRF in the cell growth and differentiation process. Finally, our present findings on CRF–MPA interaction confirm and emphasize the role of progesterone as enhancer of the effects of cAMP generating compounds in decidualization of endometrial stromal cells. In fact, the results obtained are comparable to those resulting following relaxin and MPA treatment [4].

In conclusion, the present study indicates CRF as a possible regulatory factor of decidualization of endometrial stromal cells, interacting with progestin in this cell differentiation process.

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