J. Steroid Biochem. Molec. Biol. Vol. 42, No. 3/4, pp. 337–344, 1992
Printed in Great Britain.

0960-0760/92 $5.00 + 0.00

IN VITRO DECIDUALIZATION OF HUMAN ENDOMETRIAL STROMAL CELLS

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(Received 1 August 1991)

Summary—Stromal cells isolated from proliferative human endometrium undergo morphologic and biochemical changes when exposed to a mixture of ovarian hormones, acquiring characteristics of decidual cells. In addition to the previously reported progestin-induced secretion of prolactin (PRL) by explants of human proliferative endometrium, and of PRL and laminin by stromal cells in culture, "*in vitro*" induction of several other decidual cell products was demonstrated in the present study, using cultures of stromal cells isolated from proliferative endometrium.

Incubation of stromal cells with a mixture of estradiol, medroxyprogesterone acetate and relaxin, at a concentration reported to yield maximal stimulation of PRL production, resulted in changes from elongated to rounder cells, approx. 90% of which showed immunostaining for PRL under these conditions. Immunocytochemical procedures were carried out on cytospins of decidual cells isolated from decidual tissue adherent to fetal membranes collected at delivery (positive controls), and on stromal cells cultured in Lab-Tek chamber-slides, in the absence (negative controls) or in the presence of added hormones. Antibodies to 24K (a heat-shock protein also named HRP27), desmin (present in intermediate filaments), p29 (a protein associated with the estrogen receptor), and PP12 (an insulin growth factor-1 binding protein), did not react with stromal cells isolated from proliferative endometrium but showed immunostaining of the rounder cells obtained after hormonal treatment when tested with the peroxidase-labeled second antibody complex. In another series of similar experiments, in which the same decidualization end-points were employed, changes in 24K, desmin and PP12 expression were obtained by adding to the insulin-containing medium PRL instead of the hormonal mixture, a finding suggesting sequential steps during the decidualization process.

INTRODUCTION

One of the remarkable events occurring in the human endometrium is the decidualization of the stroma. During this process fibroblast-like mesenchymal cells undergo morphologic changes and extensive biochemical differentiation reflected by the expression of a multiplicity of new products, assumed to play a still undetermined role in embryo implantation and maintenance of pregnancy. In humans, this process takes place during the normal luteal phase under the influence of progesterone, probably in concert with relaxin also secreted by the corpus luteum, even in the absence of an implanting blastocyst. In contrast, decidualization in rodents occurs only in areas adjacent to the blastocysts or requires mechanical stimulation of a hormonally prepared endometrium [1, 2]. Decidualization is also evident in non-pregnant women treated with progestins [3, 4] and its hormonal dependence can be further inferred from the extensive decidual changes observed in the endometrium during ectopic pregnancies, in the absence of intrauterine fetal tissue [5, 6].

Huang *et al.* have reported that progestins, added to an insulin-containing medium in which stromal cells isolated from proliferative endometrium are cultured, can induce the production of prolactin (PRL), a characteristic product of decidual cells, and that this action is enhanced by estradiol (E_2), likely by maintaining progesterone receptor levels. PRL production can be further augmented by addition of relaxin (Rlx), a peptide produced by the corpus luteum [8] and immunocytochemically detectable in decidual cells [9]. In addition to

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PRL, laminin and fibronectin production has been induced by the mixture of progestin and estrogen in this system, even under serum-free conditions [7, 10, 11].

In order to study the completeness of the decidualization achieved by these procedures, we utilized the same *in vitro* system and a mixture of medroxyprogesterone acetate (MPA), E_2 and Rlx, at the optimal concentrations reported by Huang *et al.* [7], searching for the formation of other products. The choice of proteins tested (PRL, 24K, desmin, p29 and PP12) was based on their reported production by decidual cells and by the availability of antibodies. The presence of 24K, also known as stress responsive protein 27 (SRP27), in human endometrium and decidual cells has been reported by Ciocca *et al.* [12–14]. Placental protein 12 (PP12) was shown by Rutanen *et al.* [15] to be synthesized by decidua from early pregnancy and to bind insulin growth factor-1 (IGF-1); this protein is identical to the α-1 pregnancy associated protein (α1-PEG) described by Bell *et al.* [16]. Desmin has been identified as an intermediate filament protein in decidual cells; Glasser *et al.* [17, 18] observed that its appearance in the rat uterus is associated to the decidualization process. A 29K protein (p29) was reported by King *et al.* [19] to be associated with the estrogen

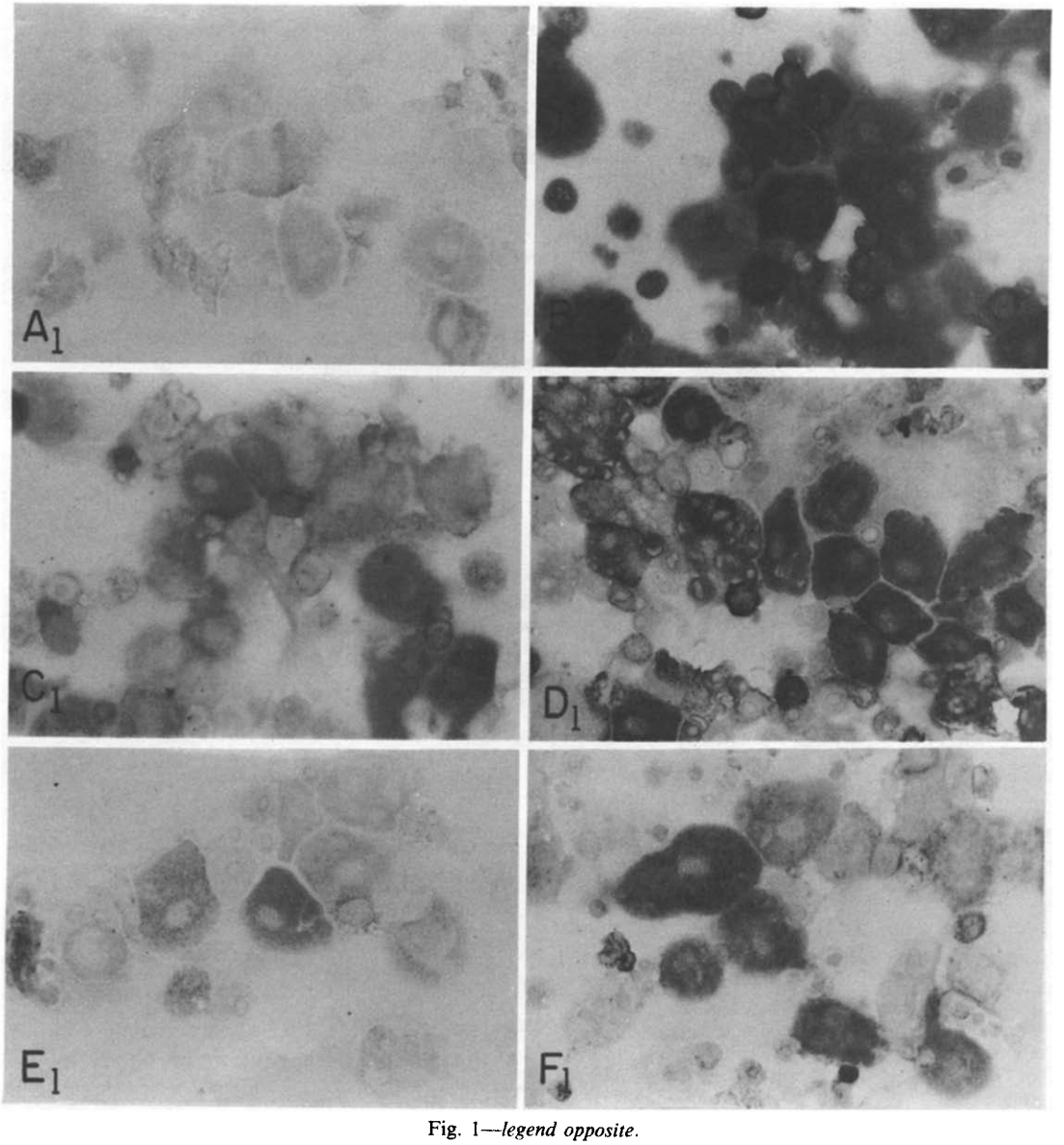

Fig. 1—*legend opposite.*

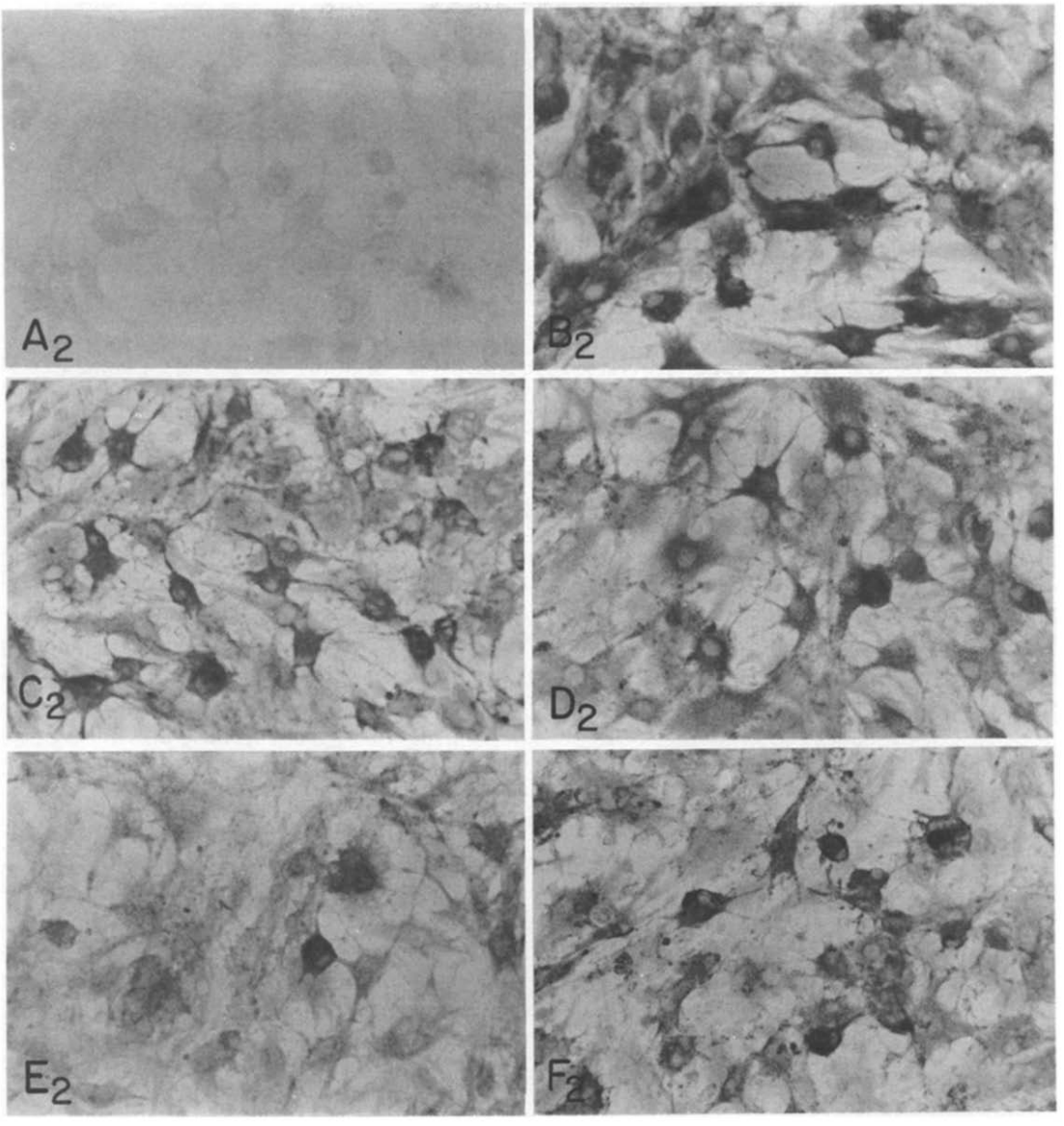

Fig. 1. Immunostaining of decidual cells adhered to fetal membranes collected at term delivery (A1 to F1) and of stromal cells decidualized "*in vitro*" (A2 to F2). Decidualized cells were assayed on slides from the Lab-Tek chambers in which they were cultured in the presence of hormones; decidual cells were tested as cytospins since they do not adhere to the slides. A1 and A2: *controls*, in which first antibody was replaced by non-immune rabbit serum (same absence of staining was obtained with non-immune mouse serum); B1 and B2: *PRL antibody*; C1 and C2: *24K protein, antibody*; D1 and D2: *desmin antibody*; E1 and E2: *p29 antibody*, F1 and F2: *PP12 antibody*. All tests were performed at the end of 5–18 days of incubation periods. No staining was observed under the same conditions in the original preparations of stromal cells from proliferative endometrium before culture or after culturing them in the absence of added hormones.

receptor and to be histochemically detectable in endometrial stromal cells at levels that increase significantly during decidualization *in vivo* [20].

The capability of PRL to act as an inducer of decidualization in medium containing insulin but in the absence of the other exogenous hormones, suggested by previously published results [21] was confirmed in the present study.

EXPERIMENTAL

Reagents

The antibodies used for these studies were commercially available or obtained through the generosity of colleagues, as follows: monoclonal antibody against PP12 (No. 6301, 6303) from Dr Frederika Pekonen (Minerva Institute of Medical Research, Helsinki, Finland),

monoclonal anti-p29 from Dr R. J. B. King (Imperial Council Research Fund Lab., London, England), monoclonal anti-24K from Dr W. L. McGuire (University of Texas, San Antonio, TX, U.S.A.), rabbit polyclonal anti-human PRL from Dr S. Raiti (National Hormone and Pituitary Program, NIADDK), polyclonal rabbit anti-human desmin from Chemicon International Inc. (El Segundo, CA, U.S.A.) and monoclonal anti-human laminin (mouse IgGl isotype) from Sigma Chemical Co. (St Louis, MO, U.S.A.). Antibodies were used at dilutions optimized for adequate staining with minimal background. Immunocytochemical procedures were performed with Vectastain ABC kit (Vector Laboratories Inc, Burlingame, CA, U.S.A.) diaminobenzidine tetrachloride (DAB) substrate (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, U.S.A.) and 30% hydrogen peroxide (Sigma).

Porcine relaxin was supplied by the National Hormone and Pituitary Program (NIADDK); E_2 and MPA were purchased from Sigma.

Tissues and cells

Endometrial tissue specimens were obtained from cycling women undergoing hysterectomy for leiomyomas. A portion of each specimen was fixed in formalin and kindly dated by Dr L. Deligdisch according to Noyes *et al.* [22]; only histologically normal proliferative endometrium was utilized. In order to isolate stromal cells, tissues were minced under sterile conditions and then exposed to 0.25% collagenase (Worthington, type I) in Ham's F10 medium (Flow Labs., Rockville, MD, U.S.A.) containing 10% charcoal-treated bovine calf-serum (ct-CS), using capped sterile glass vials under agitation at 37°C for about 2 h. The suspension of glands and dispersed cells was passed through a 38 μm stainless steel sieve to retain the glands; the collected filtrate was placed in plastic culture dishes allowing attachment for 30 min. After this period, the supernatants containing erythrocytes and other non-attaching cells were discarded. The next day the attached stromal cells, a preparation consistently found by immunocytochemical tests with cytokeratin antibodies to be free of epithelial cells, were collected by treatment with trypsin (0.05%)–EDTA (0.53 mM) in Ca^{2+}-free Hank's balanced salt solution. The cells were washed with serum-containing medium and distributed into several culture dishes for medium collection, and into several Lab-Tek chamber-slides (Nunc Inc., Neperville, IL, U.S.A.) for immunocytochemical testing. Cells were cultured in RPMI 1640 (GIBCO, Grand Island, NY, U.S.A.) containing 10% charcoal-treated fetal bovine serum (ct-FBS), 7 μg/ml insulin, and 1% of an antibiotic–anti-mycotic mixture (GIBCO), with or without a mixture of MPA (1 μM), E_2 (1 nM) and Rlx (100 ng/ml). The concentrations of MPA and Rlx were those yielding maximal stimulation according to Huang *et al.* [11]. The final concentration of ethanol added with the E_2 and MPA solutions was 0.2%. In some experiments, cells were cultured in a solution of PRL (500 ng/ml) in the medium described above, without added MPA, E_2 or Rlx. All immunocytochemical procedures were performed at the beginning and the end of the 5–18 day cultures.

Decidual cells, isolated from fetal membranes obtained after term delivery as described previously [23], were used as positive controls. In brief, the isolation procedure involved mincing decidual tissue in Ham's F10 medium containing 10% ct-CS, dispersing cells by treatment with a mixture of collagenase and DNase, repeated passages through a 22 gauge needle and separation of the PRL-producing cells on Percoll gradients. The isolated cells were washed and transferred to culture dishes. Although the cells do not attach to the dishes, they remain viable in Ham's F10 with 10% ct-FBS, as evidenced by trypan blue exclusion. In preparation for immunocytochemical tests, these cells were transferred to microscope slides by centifugation using a cytospin rotor (Shadon Southern Instruments, Sewickhy, PA, Model 2).

Immunocytochemistry

Immunocytochemical tests on stromal cells were performed on Lab-Tek slides in which they were had grown for several days as indicated for each experiment. The cells were fixed in acetone, a fixative found to be adequate for these tests, at −20°C for 5 min, rehydrated and immunoassayed for PRL, 24K, desmin, PP12, p29 and, in some experiments, laminin anti-genic sites, using the avidin–biotin immunoperoxidase method. The cells were pretreated with 0.3% hydrogen peroxide in methanol for 30 min to remove endogenous peroxidase activity, incubated with normal goat serum (or horse serum when monoclonal antibodies were used) in PBS for 1 h to saturate non-specific sites for IgG, and exposed for 1–2 h to the appropriately diluted anti-sera: 1:300 for PRL, 1:200 for 24K, 1:100 for desmin, 1:100 for PP12, 28 μg/ml for p29,

and 1:2000 for laminin, as recommended by the suppliers or determined by testing. This step was followed by sequential exposure to the following solutions: biotinylated goat anti-rabbit (or horse anti-mouse) IgG for 1 h, and avidin–biotinylated horseradish peroxidase in PBS for 1 h. After each step, the cells were rinsed in PBS for 15 min (3 times, 5 min each). Peroxidase activity was visualized by incubation with DAB (0.5 mg/ml) and H_2O_2 (0.01% v/v) in PBS for 2–7 min. The cells were then washed in water and the coverslips were mounted with Gel/Mount (Biomeda Corp., Foster City, CA, U.S.A.). The cellular outline and intracellular localization of brown chromogenic product were readily visible without counterstaining. Parallel cell preparations were stained with hematoxylin–eosin to evaluate cell morphology. All of the procedures were carried out at room temperature, only the incubation with PP12 was performed at 37°C, as indicated in the protocol made available by Dr Pekonen.

Specificity of the assays was verified by substitution of the primary anti-serum with non-immune rabbit in PBS (or mouse serum when monoclonal antibodies were used). Specificity of the reaction with the PRL anti-serum was demonstrated by preabsorbing the antibody with purified human PRL in buffer solution (1 μg/ml) overnight at room temperature, prior to starting the incubation.

Ten different fields for each slide were examined in all experiments, counting elongated and rounded cells separately. Positive staining was determined on the basis of the intracellular color contrast in the staining of nuclei, different cytoplasmic regions and background throughout the whole field. Color photographs of some fields were made and visual counting was repeated on the enlarged prints, confirming results from direct observations. Preparations stained with hematoxylin–eosin served to confirm the relative proportion of the 2 morphologic phenotypes.

PRL output into the medium

In order to evaluate PRL production by stromal cells cultured in plastic dishes in the presence or absence of the mixture of hormones, media was collected at different periods of incubation and centrifuged at 3000 *g* to separate floating cells and debris, keeping the supernatants at −20°C until radioimmunoassayed for PRL. After thawing, the solutions were concentrated 10-fold by ultrafiltration through YM-5 membranes (Amicon, Danvers, MA, U.S.A.). Levels of PRL in the concentrates were measured by double antibody RIA procedures using human PRL standards in order to estimate rates of daily production of the hormones. Dr E. Diamond, director of the Clinical Analysis Laboratories of the Division of Endocrinology, Department of Medicine at our Institution, supervised the PRL radioimmunoassays. The coefficient of variation within assays was 3–4% and between assays was 6–13%. Medium containing 10% ct-FBS, kept under the same incubation conditions in the absence of cells and concentrated as described above, did not show measurable PRL levels.

RESULTS

Figure 1 shows representative illustrations of the immunocytochemical straining for PRL, 24K, desmin, p29 and PP12. Panels A1 to F1 correspond to cytospins of decidual cells isolated from fetal membranes at term which serve as positive controls. Panel A2 to F2 correspond to stromal cells preparations cultured for 8 days in Lab-Tek slide-chambers using RPMI 1640 medium with the mixture of ovarian steroids. A1 and A2 are controls in which the first antibody was replaced by non-immune rabbit serum (similar results for negative controls obtained with non-immune mouse serum are not shown).

As reported in a previous publication [7] some of the originally elongated stromal cells became rounder and acquired immunocytochemical positivity to PRL during culture in the presence of the hormones. The strongly positive staining

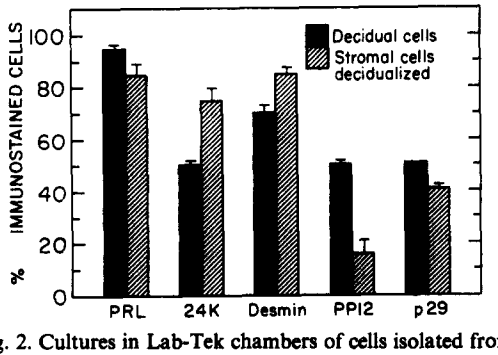

Fig. 2. Cultures in Lab-Tek chambers of cells isolated from proliferative endometrium were exposed to hormones. About 90% of the cells were morphologically converted from elongated fibroblast-like cells to a rounder phenotype. The columns correspond to the percentage of decidual cells or morphologically converted stromal cells that show immunostaining with the indicated antibodies. Standard deviations are indicated by the bars ($n = 3$).

Table 1. Summary of results on *in vitro* decidualization of stromal cells

Exp.	Histologic dating of proliferative endometrium	Exposure to hormones[a] (days)	PRL conc. at end culture[b] (mg/ml)	Morphologic change (spindle-like to round shaped)	Immunocytochemical test				
					PRL	24K	Desmin	PP12	p29
1	Late	6	102	+	+	NT	NT	NT	NT
2	Late	8	148	+	+	NT	NT	NT	NT
3	Late	10	135	+	+	NT	NT	NT	NT
4	Late	10	122	+	+	NT	+	NT	NT
5	Late	10	140	+	+	+	+	+	+
6	Late	10	134	+	+	+	+/−[c]	+	+
7	Late	18	357	+	+	+	+/−[c]	+	+
8	Mid	5	20	−	+	NT	+	NT	NT
9	Mid	10	82	+	+	+	+	+	+
10	Early	6	10	−	+	+	NT	+	+
11	Early	9	85	+	+	+	+	+	+
12	Early	10	100	+	+	+	+	+	+
13	Early	11	19	−	+	+	NT	+	+

[a]MPA (10 nM) + E_2 (1 nM) + Rlx (100ng/ml).
[b]Controls without hormones: PRL not detectable (<0.15 ng/ml).
[c]+/− control lightly positive.
NT: not tested.

obtained with the primary antibodies tested was mostly associated with the round cells. All the immunocytochemical tests performed with these antibodies were negative when applied to stromal cells from proliferative endometrium before they were placed in culture. Cells cultured for up to 18 days in the absence of hormones did not change the original spindle-like shape and did not show immunostaining.

Figure 2 indicates the fraction of morphologically "decidualized" (rounder) cells that show staining with each of the antibodies tested. The distinction between elongated or rounder cells is usually clear; under the conditions of these experiments approx. 80–90% of the cells became morphologically decidualized. We considered positive all the cells that were stained above the pale uniform background, without rating the intensity of the staining. The fraction of round cells showing immunoreactivity ranged from 90% for for PRL to 15% for PP12. A smaller proportion (30–40%) of the few elongated cells present in these preparations were also immunostained when using the PRL and 24K antibodies. The variability in the proportion of morphologically decidualized cells reacting with the different antibodies may indicate cell cycle dependent changes in the concentration of the intracellular proteins or heterogeneity among the stromal cells. The low percentage of PP12 stained cells in decidual cells isolated from term fetal membranes is consistent with the observation that this protein peaks at 9–10 week gestation [24].

These results are representative of a total of 13 experiments, listed in Table 1. Morphologic changes to rounder cells were observed in 10 of them; the 3 which did not yield clear evidence of morphologic changes under the influence of the hormonal mixture corresponded to stromal cells from 1 mid-proliferative tissue cultured for only 5 days (exp. No. 8) and from 2 early proliferative endometria cultured for 6 or 11 days (exps Nos 10 and 13, respectively). Interestingly, the failure to obtain morphologic changes was associated with cultures with the 3 lowest concentrations of PRL in the medium corresponding to the last 24 h in culture (10 to 20 ng/ml in contrast to concentrations greater than 80 ng/ml in the others). Apparently the non-quantitative immunostaining is more sensitive as an indicator of decidualization than morphologic changes.

Table 2. Immunocytochemical testing for the effects of PRL on stromal cells isolated from proliferative endometrium

Exp.	Products tested							
	Desmin		PP12		24K		Laminin	
	Control	+PRL	Control	+PRL	Control	+PRL	Control	+PRL
1	−	+	−	+	−	+	NT	NT
2	−	+	−	+	−	+	NT	NT
3	−	+	−	+	−	+	−	+
4	−	+	−	+	−	+	−	−
5	−	+	−	−	+	+	−	+

NT: not tested

Immunocytochemical detection tests for PRL under hormonal influence were positive in all 13 experiments, and tests for 24K, desmin, PP12 and p29 were positive in all 8 experiments in which each of them were tested (Table 1). Tests on cells cultured in medium without added hormones (controls) were all negative with the exception of 2 experiments (Nos 6 and 7) in which controls for desmin were positive, although less intensely stained than the cells exposed to hormones. Note that these were late proliferative endometria, one of them cultured for the longest period (18 days exp. No. 7).

Table 2 presents results from 5 experiments in which stromal cells from proliferative endometrium were incubated with PRL for 10–15 days in an attempt to obtain immunocytochemical evidence of decidualization. Conclusive results indicating PRL action (positive immunostaining of cells exposed to the test compound, negative controls) were observed in all experiments testing for desmin, in 4 out of 5 testing for PP12, in 4 out of 5 testing for 24K and in 2 out of 3 testing for laminin.

DISCUSSION

The changes in morphology and the induction of PRL production in stromal cells isolated from proliferative endometrium observed when they are cultured in medium containing a mixture of a progestin, estradiol and relaxin suggested that decidualization of these cells had been obtained. Before such a statement is justified, however, it is necessary to demonstrate the expression of other products characteristic of decidual cells. The results reported here, showing the induction of PRL, 24K, desmin, PP12 and p29, together with the already reported expression of other decidual proteins, including laminin and fibronectin [7, 10, 11], contribute to support the conclusion that full differentiation occurs *in vitro*. In rodent endometrium, endometrial stromal cells, distinct from the cells forming the deciduoma, start to synthesize and secrete basal membrane component and resemble *in vivo* decidualized cells when explanted *in vitro* [25]. Sananes *et al.* [26] have shown, however, that pretreatment of rats with progesterone is a prerequisite to obtain *in vitro* decidualization of endometrial stromal cells and indicated that the mincing of the uterus and the plating of cells may be equivalent to the mechanical stimulation necessary to provoke decidualization *in vivo*, likely through processes mediated by prostaglandins.

The possibility that the mechanism of decidualization by progestins may involve a cascade of events, following a primary induction by progesterone of key products allowing the expression of other proteins, is suggested by the observed decidualizing effects of PRL.

Note added in proof

The identity between hsp27 (24K or HRP27 in this article) and p29 has been recently reported:

Ciocca D. R. and Luque E. H.: Immunologic evidence for the identity between hsp27 estrogen-regulated heat shock protein and the p29 estrogen receptor-associated protein in breast and endometrial cancer. *Breast Cancer Res. Treat.* **20** (1991) 33–42.

Mendelsohn M. E., Zhu Y. and O'Neill S.: The 29-kDa proteins phosphorylated in thrombin-activated human platelets are forms of the estrogen receptor-related 27-kDa heat shock protein. *Proc. Natn. Acad. Sci. U.S.A.* **88** (1991) 11212–11216.

Acknowledgements—This study was made possible by the kindness of Drs F. Pekonen, R. J. B. King and W. L. McGuire who made available to us the antibodies against PP12, p29 and 24K, respectively. We are also grateful to Dr E. Diamond, Division of Endocrinology, Department of Medicine, Mount Sinai School of Medicine, NY for the measurements of PRL levels. The work was supported by Grant HD-07197, awarded by the National Institute of Child Health and Human Development, USPHS.

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