

In vitro decidualization activity assay of human endometrial stromal cells

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Summary

To address the need for a simple and reliable system for evaluating the effects of bioactive substances on decidualization of endometrial stromal cells, we designed and tested an assay for measuring decidualization activity. With our assay, it is easy to examine the effects of various bioactive substances on endometrial stromal decidualization. For example, as shown in this study, it is now clear that neither RANTES nor gonadotropin releasing hormone agonist has any effect on endometrial stromal decidualization. Our simple assay has also made it possible to evaluate individual stromal decidualization activities and the regulation of decidualization.

Key words: Decidualization; Endometrial stromal cells; RANTES; GnRH agonist; Endometriosis.

Introduction

Remodeling of human endometrial tissues is regulated by proliferation and differentiation (or decidualization) of endometrial stromal cells (ESCs) although the regulatory mechanisms of stromal differentiation remain unclear. Both types of endometrial stromal cells, non-decidualized and decidualized, produce various cytokines such as interleukin-1 (IL-1) [1-3], transforming growth factor- β (TGF- β) [4-7], epidermal growth factor (EGF) [8-10], and hepatocyte growth factor (HGF) [11, 12]. These stromal cytokines are considered to regulate proliferation, differentiation, and apoptotic susceptibility of endometrial epithelial cells, endometrial endothelial cell growth, and embryo implantation. However, direct evidence of these regulations has been minimal. Moreover, endometrial stromal cytokines are hypothesized to regulate proliferation and differentiation of the endometrial stromal cells by themselves through autocrine mechanisms, although such regulation also has seldom been reported.

There have been several reports elucidating induction systems of in vitro decidualization [13-20] because culturing of ESCs for a long time has been easy; however, only a few reports have described the effects of cytokines on in vitro decidualization [15, 19, 21-23]. There are a few reasons why cytokine effects on ESCs have rarely been investigated. It is difficult to obtain sufficient quantities of human ESCs for multiple culturing. It is difficult to count viable cell numbers of ESCs by ^3H -thymidine uptake procedure because of the contact inhibition character of ESCs. No simple relatively fast cell culture system for inducing endometrial stromal decidualization has been established.

Recently identified cytokines such as interleukin 11 (IL-11) [24], leukemia inhibitory factor (LIF) [25], and vascular endothelial cell growth factor (VEGF) [26] have

also been reported to be produced by ESCs, although the effects of these new cytokines on proliferation and differentiation of ESCs have not been elucidated. There has been a great need for a new simple assay system for evaluating the effects of any bioactive substance on ESC decidualization. Therefore, we chose to undertake the design and testing of such an assay of in vitro decidualization activity. We examine, here, the direct effects of RANTES, a T cell derived cytokine, which was reported to be elevated in the ascitic fluid of endometriotic patients [27], and the effects of a gonadotropin releasing hormone agonist (GnRHa), buserelin acetate, which is an antiendometriotic medicine.

Material and Methods

In vitro culture of human endometrial stromal cells

ESCs used in this study were cultured in OPTI-MEM, 5% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 mg/ml) (all from GIBCO-BRL, Gaithersburg, MD, USA), and 17β -estradiol (10 nM) (Sigma, St. Louis, MO, USA). Human ESCs were obtained with informed consent from patients whose uterus was being surgically excised due to uterine leiomyoma and the ESCs were cultured as follows. Endometrial tissues were cut into small fragments with scissors and incubated with a medium containing 0.1% w/v collagenase (Wako-Chemicals, Tokyo, Japan) for 30 minutes at 37°C. The digested tissues were then centrifuged at 1500 rpm for 6 minutes at 4°C. The cell pellets were resuspended in the estradiol-containing medium, filtered with a 40- μm nylon mesh filter to remove epithelial cells, and then cultured. Non-adherent cells were removed by several intensive washings over 2 weeks. Finally more than 98% of the residual adherent cells were identified as vimentin-positive ESCs. Those cells were used for the in vitro decidualization experiments.

In vitro decidualization of human endometrial stromal cells

ESCs were detached in 0.25% trypsin/1 mM EDTA (GIBCO-BRL) and cultured until confluence in 96-well culture plates. To induce in vitro decidualization, the cells were then cultured with

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0.5 mM 8-Br-cAMP (Sigma), a decidualization inducer, for 10 days. The optimum concentrations of the decidualization inducers were determined in a previous study of ours [20]. To examine the dose-dependent effects of RANTES (IBL, Maebashi, Japan) or buserelin acetate (courtesy of Hoechst Marion Roussel Japan, Tokyo, Japan) on decidualization, various concentrations of recombinant human RANTES or buserelin acetate were added to the medium during or after decidualization. The culture medium was changed every 3 days, and the supernatants were stored at -20°C to assay prolactin (PRL) concentration. Viable cell numbers were assayed with a cell proliferation assay kit on day 13. Figures 1 and 2 represent prolactin accumulated in culture supernatants from days 10 to 13. Each bar represents the mean \pm SEM of three experiments. Significance was set at $p < 0.05$. All the concentrations were tested in triplicate, and experiments were repeated more than three times to verify the results.

Enzyme immunoassay of PRL released into culture supernatant

PRL concentrations of the culture supernatants were assayed with an enzyme immunoassay instrument, SR-1 (Biochem. Immunosystems, Rome, Italy). All experiments were performed in triplicate as described above. Data was analyzed with the Student's t-test ($n=3$).

Viable cell assay

Viable cell numbers were assayed with a non-RI colourimetric assay kit, XTT (Boehringer-Mannheim, Mannheim, Germany), which performs a quantitative assay of enzyme activity in viable cells in terms of absorbances at 450 nm. Our preliminary studies showed that the absorbance at 450 nm was proportional to viable cell numbers (data not shown). The inhibitory effect of RANTES and buserelin acetate on the cell viability of ESCs was assayed as follows. ESCs were detached in 0.25% trypsin/1 mM EDTA (GIBCO-BRL) and cultured until confluence in an estradiol-containing medium in 96-well culture plates (2500 cells/well). On day one, 0.5 mM 8-Br-cAMP and various concentrations of RANTES or buserelin acetate were added to the media. The culture media were changed every 3 days, and on day 13, viable cells were counted. To examine the effects of RANTES or buserelin acetate on decidualized cell viability, ESCs were cultured with 0.5 mM 8-Br-cAMP without RANTES or buserelin acetate for 10 days, during which the culture medium was changed every 3 days. On day 11, various concentrations of RANTES or buserelin acetate were added to the cultures, and on day 13, viable cell numbers were assayed. All the experiments were performed more than three times to verify the results. The data are expressed as mean \pm SEM and were analyzed with the Student's t-test ($n=5$).

Results

Neither RANTES nor GnRHa had any inhibitory effect on viability of the cAMP-stimulated ESCs within 2 weeks (Figure 3). Nor did RANTES or GnRHa have any significant effect on cell viability of the decidualized ESCs (Figure 4).

Assay of PRL concentration in culture supernatant can evaluate decidualization activity because PRL is produced by decidualized ESCs but not by non-decidualized ESCs [1]. RANTES and GnRHa did not show any significant effects on PRL release from 8-Br-cAMP-stimulated cells (Figure 1). This indicates that neither RANTES nor GnRHa has any effect on stromal decidualization.

Effects of RANTES and GnRHa on PRL release from the decidualized ESCs were also examined (Figure 2). Various concentrations of RANTES or GnRHa did not show any effect on PRL release from the decidualized cell.

Discussion

We have established a simple in vitro assay of decidualization activity in ESCs. This in vitro assay system has the following characteristics: 1) decidualization activity can be assayed using small numbers of ESCs because 96-well culture plates are used in this decidualization culture; 2) many decidualization experiments can be performed simultaneously to evaluate effects of many bioactive substances on ESCs; 3) individual decidualization activity can be evaluated. Decidualization of ESCs can be quantitated by the assay of PRL concentrations in the culture supernatants. In the present culture system, PRL concentrations of more than 50 samples can be assayed within 2 hours with only 100 μl of each culture supernatant. The lowest limit of the assay is 5 ng/ml of PRL. Since this assay system can measure changes in PRL concentrations during decidualization, the time course of stromal decidualization can be evaluated by this assay. Previously, no simple assay has been available for evaluating the decidualization process. With the 96-well culture plate coated with extracellular matrices used in this assay system, effects of cell adhesion between ESCs and the extracellular matrices can be also quantitated.

Since proliferation of ESCs is inhibited by contact inhibition when the normal adherent cells are cultured on plates, viable cell numbers of ESCs can not be measured precisely by the ^3H -thymidine uptake method, a DNA synthesis assay. In this study, we used a non-RI viable cell assay kit, which is based on measuring mitochondrial enzyme activity in viable cells. With this assay, therefore, viable cell numbers can be counted, and the effects of various cytokines on ESCs can be evaluated even in a small cell culture system such a 96-well plate culture. Since this assay does not need radioisotopes and exfoliating cells from culture flasks, it is a safe and easy method of determining viable cell numbers. However, this assay can not be used to examine enhancing effects on cell proliferation in a long-term culture because the contact inhibition phenomenon would develop over time.

Kokawa *et al.* [28] reported that DNA fragmentation in human endometrium is enhanced during the peri-menstrual period and that immunohistochemical apoptotic changes are widely distributed in decidualized endometrial stromal cell immediately before menstruation. From their results, they concluded that human menstruation can be initiated by endometrial stromal apoptoses. However, we could not detect any apoptotic changes within 2 weeks in ESC cultures decidualized by 8-Br-cAMP. In vitro decidualized ESCs could survive even when the decidualized cells were cultured in the medium without estradiol or 8-Br-cAMP (data not shown). These results suggest that decidual cells and ESCs do not become

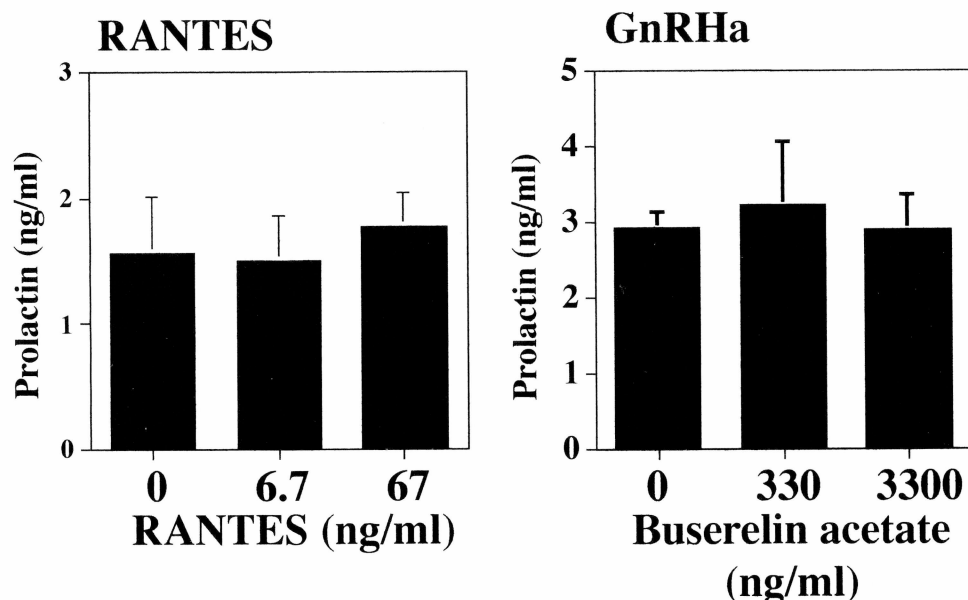


Figure 1. — Effects of RANTES and GnRHa on prolactin release from human endometrial stromal cells co-stimulated with 8-Br-cAMP.

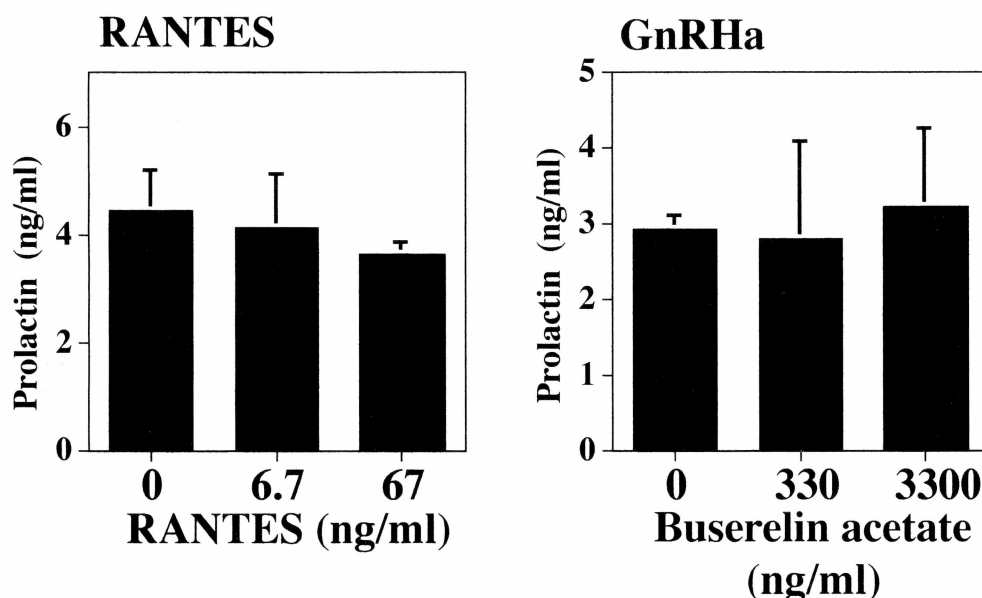


Figure 2. — Effects of RANTES and GnRHa on prolactin release from 8-Br-cAMP-induced decidualized cells.

apoptotic cells spontaneously. If premenstrual decidual cells become apoptotic, which was suggested by Kokawa *et al.*, the apoptosis may be caused by unknown apoptotic inducers, not by deprivation of stimulants such as steroids or cAMP-inducing substances. This means that an increase in unknown apoptotic inducers or enhanced apoptotic susceptibility may occur in premenstrual ESCs. Our present experimental system can be applicable for examining whether menstruation may be induced by endometrial stromal apoptoses.

Although increased RANTES in the ascitic fluid of endometriotic patients has been reported [27], the effects of RANTES on ESCs have not been analyzed yet. Our results suggest that increased RANTES in endometriotic patients does not have any significant effects on cell growth suppression and decidualization of ESCs. Buserelin acetate (GnRHa) is considered to have two endocrine mechanisms; direct effects on target cell GnRH receptors and secondary effects via hypothalamic GnRH receptors. Our present study indicates that GnRHa does

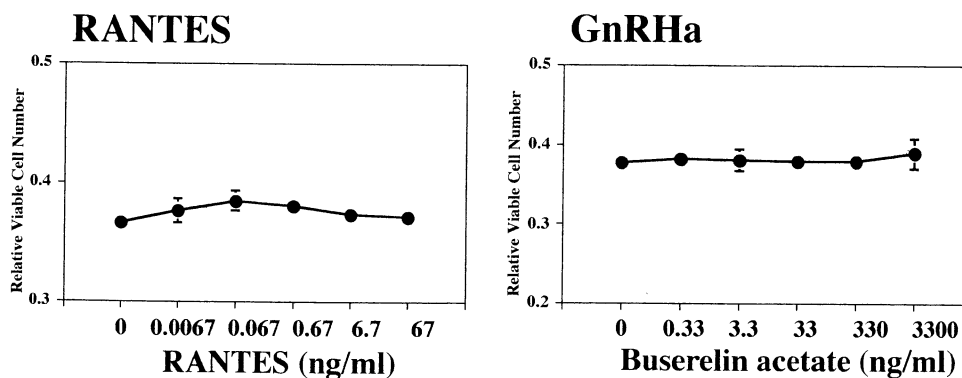


Figure 3. — Effects of RANTES and GnRHa on viable cell numbers of human endometrial stromal cells co-stimulated with 8-Br-cAMP.

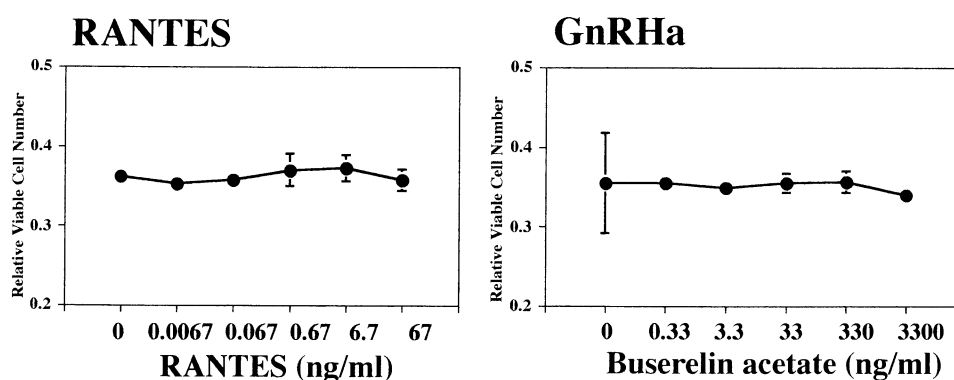


Figure 4. — Effects of RANTES and GnRHa on viable cell numbers of 8-Br-cAMP-induced decidualized cells.

not have any direct inhibitory effects on the proliferation and decidualization of ESCs.

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