Suppression of interleukin 8 production by progesterone in rabbit uterine cervix

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Uterine cervical fibroblasts prepared from rabbits at 23 days of gestation were found to produce spontaneously the neutrophil chemotactic factor/interleukin 8 (IL-8). When the cells were treated with recombinant human interleukin 1 α and 1 β (rhIL-1 α and -1 β), both cytokines similarly enhanced the production of IL-8 in a dose-dependent manner. Recombinant tumour necrosis factor α also enhanced its production to a lesser extent, but interleukin 6 failed to modulate the production. Physiological concentrations of progesterone suppressed both the spontaneous and IL-1-mediated production of IL-8 in parallel with the decrease in the steady-state levels of its mRNA. These suppressive actions of progesterone were offset by co-treatment of cells with

a progesterone antagonist, mifepristone (RU486). In conclusion, basal and IL-1-induced IL-8 production in rabbit uterine cervical fibroblasts is down-regulated by progesterone at the transcriptional level. These results obtained *in vitro* and our previous observations indicating that progesterone modulates the extracellular matrix breakdown via the suppression of production of matrix metalloproteinases and the augmentation of production of their specific inhibitors (TIMP-1) [Sato, Ito, Mori, Yamashita, Hayakawa and Nagase (1991) *Biochem. J.* 275, 645–650] may explain the mechanisms of the maintenance of pregnancy until parturition and the acceleration of uterine cervical ripening and dilatation at term.

INTRODUCTION

Infiltration of neutrophils into the stromal tissue is one of the characteristic changes observed in the ripened and dilated uterine cervix [1]. The leucocytes in the cervix are considered to release locally cytokines, including interleukin 1 (IL-1), chemotactic factors, matrix metalloproteinases (MMPs) and leucocyte elastase, which are closely connected with cervical ripening and dilatation [1–4]. It is also well known that leucocytes enhance the production of inflammatory cytokines and MMPs by tissues. It is therefore suggested that neutrophil chemotactic factor(s) which recruits leucocytes may play significant roles in cervical ripening and dilatation at term. In this regard, we have previously reported that the rabbit uterine cervix at term has high levels of chemotactic activity as compared with the cervix of non-pregnant animals [5]. However, the origin and properties of this chemotactic activity in the rabbit cervix are unclear.

Interleukin 8 (IL-8) is a novel potent chemotactic cytokine for neutrophils [6] and T lymphocytes [7]. At present, various types of cells, including monocytes/macrophages, dermal fibroblasts, endothelial cells, keratinocytes, mesangial cells and human tumour cell lines, are known to produce IL-8 [6,8–11], strongly suggesting that the neutrophil chemotactic factor in the rabbit uterine cervix is IL-8 itself or a related molecule.

In the present study, we investigated the regulation of the production and immunological characterization of a neutrophil chemotactic factor produced by rabbit uterine cervical fibroblasts, and demonstrated that the chemotactic factor is characterized as rabbit IL-8, and progesterone is a potent inhibitor of both spontaneous and IL-1-mediated production of IL-8 in rabbit uterine cervical fibroblasts. These observations suggest that both IL-8 and progesterone may be potent endogenous

regulators of neutrophil recruitment into the rabbit cervix during cervical ripening and dilatation.

MATERIALS AND METHODS

Materials

The following reagents were obtained commercially: Eagle's minimum essential medium (MEM) from Grand Island Biochemical Co., Grand Island, NY, U.S.A.; alkaline phosphatase-conjugated rabbit anti-(guinea-pig IgG) IgG from BioMakor, Rehovot, Israel; fetal-bovine serum from Whittaker M.A. Bio-products Inc., Walkersville, MD, U.S.A.; rabbit anti-(mouse IgG) IgG, Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate from Sigma Chemical Co., St. Louis, MO, U.S.A. Recombinant human IL-1 α (rhIL-1 α ; 2×10^7 units/mg) and tumour necrosis factor α (rhTNF α ; 2.55×10^6 units/mg) were kindly supplied by Dainippon Pharmaceutical Co., Suita, Osaka, Japan. Recombinant human IL-1 β (rhIL-1 β ; 2×10^7 units/mg) was generously given by Otsuka Pharmaceutical Co., Tokushima, Japan. Recombinant human IL-6 (rhIL-6) was a product of R & D Systems, Minneapolis, MN, U.S.A. Recombinant rabbit IL-8, rabbit IL-8 cDNA and mouse monoclonal antibody to human IL-8 were prepared as described previously [12,13]. The anti-progesterone mifepristone (RU486) was kindly supplied by Roussel UCLAF, Romainville, France. Other reagents used were the same as in a previous paper [14].

Cell culture of rabbit uterine cervix

Uterine cervixes were removed from rabbits (Nippon white) at 23 days of gestation under sterile conditions, cleaned of fatty tissues and then washed with Ca²⁺- and Mg²⁺-free PBS [PBS(–)]

Abbreviations used: rhIL-1, recombinant human interleukin 1; IL-6, interleukin 6; IL-8, interleukin 8; TNF α , tumour necrosis factor α ; MMP, matrix metalloproteinase; MEM, Eagle's minimum essential medium; LAH, lactalbumin hydrolysate.

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containing 200 units/ml penicillin G and 200 μ g/ml of streptomycin. The finely minced tissue was digested with bacterial collagenase and the cervical fibroblast-like cells obtained were maintained in the culture in MEM/10% (v/v) fetal-bovine serum as described previously [14,15]. In most experiments, cells up to the third passage were used. To estimate the production of chemotactic factor, the culture medium was changed to MEM/0.2% (w/v) lactalbumin hydrolysate (LAH) after confluence, as described previously [15]. The harvested culture media were stored at -20°C until use.

E.I.s.a. for rabbit IL-8

Immunoreactive rabbit IL-8 in culture media was estimated by e.i.s.a. with mouse monoclonal antibody to human IL-8 which was cross-reactive with rabbit IL-8, guinea-pig anti-(rabbit IL-8) antibody and alkaline phosphatase-conjugated rabbit anti-(guinea-pig IgG) IgG as described previously [12].

Western blotting for rabbit IL-8

The sample (1.5 ml) collected from triplicate wells was first concentrated with trichloroacetic acid (final concn. 3.3%, w/v) and then subjected to SDS/PAGE with 16.5% (w/v) acrylamide slab gels [16] under reducing conditions, and then electrotransferred to a nitrocellulose filter. The filter was made to react with a monoclonal anti-(human IL-8) antibody, which was then complexed with alkaline phosphatase-conjugated rabbit anti-(mouse IgG) IgG. Immunoreactive IL-8 was detected indirectly with 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium, as described previously [17].

Extraction of RNA and slot-blot analysis

Confluent uterine cervical cells in 100 mm-diameter dishes were treated with 15 ml of MEM/0.2% (w/v) LAH containing progesterone and/or rhIL-1 α . After 24 h of treatment, total cytoplasmic RNA was isolated with a commercial RNA extraction kit (Nippon Gene Co., Tokyo, Japan) according to the manufacturer's instructions. Total RNA was denatured with formaldehyde and then transferred on to a nylon membrane (Gene Screen; DuPont, Boston, MA, U.S.A.). Slot-blot analysis was carried out by the method of Wahl [18]. Membranes were hybridized with ^{32}P -labelled nick-translated IL-8 cDNA at 42°C in $10\times$ Denhardt's solution/ $5\times$ SSPE ($1\times$ SSPE is 0.18 M NaCl/0.01 M NaH_2PO_4 /1.1 mM disodium EDTA) containing 0.1% (w/v) SDS and 150 μ g of heat-denatured salmon sperm DNA/ml. After hybridization, the membrane was washed four times in $1\times$ SSC (0.15 M NaCl/0.015 M sodium citrate, pH 7.0)/0.1% (w/v) SDS, and then washed in $0.1\times$ SSC/0.1% SDS at room temperature for 30 min and exposed to Kodak X-Omat film at -80°C .

RESULTS AND DISCUSSION

Effects of cytokines on the production of IL-8 in rabbit uterine cervical fibroblasts

We have previously reported that the rabbit uterine cervix at term contains high levels of neutrophil chemotactic activity as compared with that of non-pregnant animals [5], but the cells producing it and their properties have not yet been characterized. We therefore first examined whether or not rabbit uterine cervical fibroblasts produce rabbit chemotactic factor/IL-8. When the cells were treated with rhIL-1 α , rhTNF α and rhIL-6, rhIL-1 α increased the accumulation of immunoreactive IL-8 in the culture

media, as shown in Figure 1 (lane 2). The molecular mass of immunoreactive IL-8 in culture media was about 8 kDa, almost identical with that of recombinant rabbit IL-8 (Figure 1, lane 5). In view of these results, it is very likely that the rabbit uterine cervical fibroblast is an IL-8-producing cell, and the chemotactic factor that we previously found in rabbit uterine cervix [5] corresponds in part to rabbit IL-8. rhTNF α also augmented the apparent accumulation of IL-8 by these cells, although the extent was less than with rhIL-1 α . However, rhIL-6 did not exert any effect on the accumulation. In addition, isoforms of rhIL-1 α and -1 β equally enhanced the accumulation of immunoreactive IL-8 in a dose-dependent manner; a maximum 7-fold increase in IL-8 accumulation, in treated cells as compared with untreated

Figure 1 Effects of cytokines on the production of IL-8 in rabbit uterine cervical cells

Confluent rabbit uterine cervical cells at the first passage in 24-multiwell plates were treated with rhIL-1 α (1 ng/ml), rhTNF α (1 ng/ml) and rhIL-6 (20 ng/ml) in 1.0 ml of 0.2% LAH/MEM for 48 h. Samples (1.2 ml) of harvested and mixed culture media from quadruplicate wells were mixed with $\frac{1}{5}$ vol. of 20% trichloroacetic acid. The resultant precipitates were dissolved in 15 μ l of reducing SDS/PAGE sample buffer, and then a portion (12 μ l) was subjected to SDS/PAGE with 16.5% (w/v) acrylamide. Immunoreactive IL-8 was detected by immunoblotting as described in the text. Three independent experiments were highly reproducible, and typical data are presented. Lanes: 1, control; 2, rhIL-1 α ; 3, rhTNF α ; 4, rhIL-6; 5, standard recombinant rabbit IL-8 (20 ng). Arrow indicates the position of immunoreactive IL-8 (8 kDa).

Figure 2 Dose-dependent enhanced production of IL-8 by rhIL-1 in rabbit uterine cervical cells

Confluent uterine cervical cells at the first passage in 24-multiwell plates were treated with various concentrations of rhIL-1 α or -1 β in 1.0 ml of 0.2% LAH/MEM for 48 h. Other experimental conditions were the same as in Figure 1. The relative amounts of IL-8 were quantified by densitometric scanning and calculated as a relative induction compared with the control. Three independent experiments were highly reproducible, and typical data are shown. Lanes 1, control; 2–6 and 7–11, cells treated with rhIL-1 α or -1 β (0.001, 0.01, 0.1, 0.5 and 1 ng/ml respectively).

Figure 3 Suppression of the production of IL-8 by progesterone in rabbit uterine cervical cells

Confluent uterine cervical cells at the first passage in 24-multiwell plates were treated with or without progesterone and/or rhIL-1 α in 1.0 ml of 0.2% LAH/MEM for 48 h, and then harvested culture media were subjected to e.l.i.s.a. as described in the text. Data are means $\pm$ S.D. for three wells. Two independent experiments were highly reproducible, and typical data are shown. Cervical cells treated with (Δ) or without ($\circ$) rhIL-1 α . * and **, significantly different from respective untreated control ($P < 0.05$ and $P < 0.001$ respectively); † significantly different from rhIL-1 α -untreated control ($P < 0.001$).

P (1 μ M)	-	-	+	+	-	+	+
IL-1 (1 ng/ml)	-	-	-	-	+	+	+
RU486 (1 μ M)	-	+	-	+	-	-	+

Figure 4 Effect of the progesterone antagonist mifepristone/RU486 on the production of IL-8 in rabbit uterine cervical cells

Confluent uterine cervical cells at the first passage in 24-multiwell plates were treated with or without progesterone (P), rhIL-1 α and/or RU486 in 1 ml for 48 h. Other experimental conditions were the same as described in Figures 1 and 2. Three independent experiments were highly reproducible and typical data are presented.

cells, was observed with 1 ng/ml of both rhIL-1 α and -1 β , as shown in Figure 2.

Suppression of the production of IL-8 by progesterone in the rabbit uterine cervix

In rabbit uterine cervical fibroblasts, the production of pro-MMP-1 (procollagenase) and pro-MMP-3 (prostromelysin-1) and the expression of their mRNAs are co-ordinately suppressed

Figure 5 Effect of progesterone, rhIL-1 α and RU486 on the level of IL-8 mRNA in rabbit uterine cervical cells

Confluent uterine cervical cells at the first passage in 100 mm-diameter dishes were treated with various reagents for 24 h. Total RNA (10 μ g) was applied to nylon membrane with a slot-blot apparatus as described in the text. The relative expression of IL-8 mRNA was quantified by densitometric scanning of the autoradiograms, taking the untreated control as 100%. Two independent experiments are highly reproducible and typical data are shown. Lanes: 1, control; 2, progesterone (1 μ M); lane 3, rhIL-1 α (1 ng/ml); lane 4, rhIL-1 α (1 ng/ml) plus progesterone (1 μ M); lane 5, RU486 (1 μ M); lane 6, progesterone and RU486 (1 μ M each) and lane 7, rhIL-1 α (1 ng/ml) plus progesterone (1 μ M) plus RU486 (1 μ M).

at the pre-translational level of progesterone, as reported previously [15]. Thus the effect of this hormone on the production of IL-8 in these cells was examined by e.l.i.s.a. Confluent uterine cervical cells were found to produce constitutively immunoreactive IL-8 (about 4.5 ng/10⁵ cells per 48 h), and the IL-8 level was enhanced about 10-fold by treatment of cells with 1 ng/ml rhIL-1 α , as shown in Figure 3. When uterine cervical cells were treated with progesterone, both spontaneous and IL-1-mediated accumulations of IL-8 were similarly suppressed in a dose-dependent manner; both levels were decreased to about a maximum of 40% by 1 μ M progesterone. It is noteworthy that the suppression by progesterone of both basal and IL-1-mediated IL-8 production was effectively interfered by the progesterone-receptor antagonist mifepristone/RU486, as shown in Figure 4, suggesting that the above suppression by progesterone of IL-8 accumulation specifically appeared via its receptor.

We next examined whether the decrease in IL-8 protein level by rhIL-1 α and/or progesterone was associated with changes in its mRNA level. The steady-state level of its mRNA was measured by slot-blot analysis. As shown in Figure 5, rhIL-1 α significantly increased the steady-state level of rabbit IL-8 mRNA as compared with the untreated control (Figure 5, lane 3). The IL-1-mediated increase in IL-8 mRNA was suppressed by progesterone

(Figure 5, lane 4), whereas the suppression by progesterone was offset by the co-treatment of cells with rhIL-1 α and RU486 (Figure 5, lane 7). The steady-state levels of IL-8 mRNA in non-treated control cells were similarly modulated by progesterone and/or RU486. Thus the changes in IL-8 protein levels in rabbit uterine cervical cells closely reflected those of IL-8 mRNA.

Although it is generally known that various species of intact cells produce an insignificant amount of IL-8 [6,11], our uterine cervical cells prepared from pregnant rabbits at 23 days of gestation or at term spontaneously produced a significant amount of IL-8. It is therefore very likely that the IL-8-producing pathway in uterine cervical fibroblasts of pregnant rabbits has been activated spontaneously. This hypothesis might be further supported by our preliminary observation that cells of non-pregnant rabbits could produce less IL-8 than could those of pregnant ones (results not shown).

Concerning the regulator of IL-8 production, most attention has been paid to the inducer and/or enhancer of IL-8 production. In fact, cytokines such as IL-1 and TNF α are known to enhance the production of IL-8 in various cell types, including endothelial cells [8], human dermal fibroblasts and keratinocytes [10] and gastric cancer cells [11]. We have also demonstrated that IL-1-promoted IL-8 production with a concomitant increase in its steady-state level of mRNA in rabbit cervical fibroblasts. On the other hand, the suppressive regulator for IL-8 production has not been understood well. In rabbit uterine cervical fibroblasts, physiological concentrations of progesterone effectively suppressed both basal and IL-1 mediated production of IL-8 in accordance with the decrease in the steady-state amount of its mRNA. It was recently reported that progesterone suppresses the production of IL-8 by human chorionic decidua cells, but the change in its mRNA level was not examined [19]. We therefore in this report provide the first evidence that uterine cervical cells can produce neutrophil chemotactic factor/IL-8, spontaneously and in response to IL-1, and that progesterone is a suppressive regulator of the production of IL-8 and its mRNA. Although we reported that pharmacological concentrations of oestradiol-17 β (< 1 μ M) suppressed the production of pro-MMP-3 in rabbit uterine cervical fibroblasts [15], these concentrations of oestradiol-17 β did not suppress IL-8 production (results not shown). These different effects of oestradiol-17 β were not well understood.

In conclusion, our present finding that progesterone down-regulates IL-8 production as well as pro-MMPs [15] suggest the active participation of progesterone in the maintenance of pregnancy. In this regard, an interesting observation is reported: in the uterine cervix of pregnant guinea pigs, three progesterone antagonists induce the neutrophil invasion and connective-tissue

remodelling which might be mainly induced by MMPs [20]. In addition, the fact that the administration of the anti-progesterone mifepristone/RU486 accelerates cervical dilatation in pregnant and non-pregnant women is reported [21]. We previously reported that the rabbit uterine cervix at term contains significant levels of IL-1-like activity [3], by which the production of pro-MMPs and IL-8 might be augmented. It is therefore indicated that progesterone plays important roles in the maintenance and fine tuning of functions of the uterine cervix during pregnancy and at parturition by decreasing the production of IL-8 and pro-MMPs.

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