Induction of the synthesis of the pregnancy-specific protein p70 in the endometrium by intramuscular injection of recombinant bovine interferon- α_1 in nonpregnant ewes

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Summary. We examined the effect of recombinant bovine interferon- α_1 (rboIFN- α_1) or recombinant bovine trophoblast protein-1 (rbTP-1) on protein synthesis by endometrial explants from Day-13 cyclic ewes and studied the ability of rboIFN- α_1 injected i.m. to influence subsequent protein secretion by endometrial tissue explants.

In Expt 1, ewes were injected with either 2 mg rboIFN- α_1 or vehicle alone at 12-h intervals beginning on Day 11 of the oestrous cycle and ending on the morning of Day 13; 8 h after the last injection, ewes were hysterectomized and endometrial explant cultures were prepared. Explants were cultured for 24 h in leucine-deficient medium supplemented with 250 μ Ci L-[3 H]leucine per culture. For Expt 2, additional explants were prepared from Expt 1 controls. Explants were cultured in the presence of 0, 20 or 200 ng/ml of either rboIFN- α_1 or rbTP-1 for 24 h in leucine-deficient medium supplemented with 250 μ Ci L-[3 H]leucine per culture. Secreted proteins were analysed by two-dimensional electrophoresis and fluorography. There was a marked enhancement of a 70 kDa acidic protein, p70, in explants cultured in the presence of rboIFN- α_1 or rbTP-1. This polypeptide is a product of the gravid uterine horn from Day 14 to Day 20 of pregnancy and is a useful marker of the action of interferon- α (IFN- α) on endometrium. Enhanced production of p70 also occurred in ewes injected i.m. with rboIFN- α_1 . These results are consistent with the hypothesis that injected rboIFN- α_1 exerts its effects on such variables as interoestrus interval and maternal recognition of pregnancy by actions on the uterus.

Keywords: cattle; interferon- α ; pregnancy; protein; sheep; trophoblast

Introduction

The trophoblast interferons bovine trophoblast protein-1 (bTP-1) and ovine trophoblast protein-1 (oTP-1) are considered to play a key role in maintaining the functionality of the corpus luteum during early pregnancy in cattle and sheep, respectively (Roberts, 1989; Roberts *et al.*, 1990a). These proteins are first secreted in large amounts by the trophoblast just before the corpus luteum would normally begin to regress during a normal oestrous cycle (Godkin *et al.*, 1982; Bartol *et al.*, 1985; Hansen *et al.*, 1985). Their interaction with the endometrium appears to attenuate the frequency and magnitude of pulsatile prostaglandin F-2 α release by the uterus (Bazer *et al.*, 1986; Vallet *et al.*, 1987), even though in ewes the total quantity of PGF-2 α leaving the uterus may increase during pregnancy (Zarco *et al.*, 1988). Because of this change in uterine PGF-2 α dynamics, oTP-1 and bTP-1 have generally been considered to affect the uterus and not the ovary or some other extrauterine site. There has been no evidence to suggest that oTP-1 injected into the uterine lumen is able to escape and enter the maternal blood circulation (Godkin *et al.*, 1984).

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It was of interest, therefore, that recombinant bovine interferon- α_1 (rboIFN- α_1) injected intramuscularly into ewes was able to extend the interoestrus interval almost as effectively as when it was introduced directly into the uterus (Plante *et al.*, 1989). Even more intriguing was that intramuscular injections of this same interferon (IFN) twice a day into ewes between Days 11 and 18 after mating significantly increased their chances of maintaining a successful pregnancy (Nephew *et al.*, 1990; Roberts *et al.*, 1990b; Schalue-Francis *et al.*, 1991). In these pregnancy studies it was assumed that the injected IFN had supplemented the action of endogenous oTP-1 produced by the conceptus and had enhanced maternal recognition of pregnancy. However, there was no evidence that the biological effects of the injected IFN were manifested through its action on the endometrium. Schalue-Francis *et al.* (1991) were not able to detect antiviral activity in the uterine flushings of cyclic ewes injected with IFN. In the present study, we tested whether injected IFN influenced protein synthesis by the endometrium. We examined whether a 70 000 M_r acidic protein (p70) was induced by the treatment. This protein is produced by endometrial tissue explants and cultured epithelial cells from cyclic ewes when exposed to oTP-1 or a human IFN- α_1 (Godkin *et al.*, 1984; Vallet *et al.*, 1987; Salamonsen *et al.*, 1988; Silcox *et al.*, 1988). It is also a product of endometrial tissue derived from Day 14, 16 and 17 pregnant ewes, but not of cultured explants from cyclic ewes at equivalent stages of their oestrous cycles (Sharif *et al.*, 1989). It appears, therefore, to be pregnancy-specific as well as a sensitive marker of IFN- α action on the endometrium.

Materials and Methods

Materials

The rboIFN- α_1 (Capon *et al.*, 1985) was provided by Ciba Geigy Ltd (Basel, Switzerland) as a sterile-formulated, succinate-buffered lyophilizate containing mannitol. The placebo consisted of the same formulation minus rboIFN- α_1 . The dried substances were reconstituted with sterile, pyrogen-free distilled water. Recombinant bTP-1 was produced in our laboratory (Klemann *et al.*, 1990). Material for injection was prepared by using the placebo as diluent. L-[3,4,5- 3 H]leucine (specific activity 153 Ci/mmol) was purchased from New England Nuclear, Boston, MA, USA. Tissue culture supplies were obtained from Fisher Scientific, St Louis, MO, USA. Material used for medium preparation and two-dimensional polyacrylamide gel electrophoresis and fluorography were obtained as described by Sharif *et al.* (1989).

Medium

Eagle's minimum essential medium (MEM) was prepared deficient in L-leucine according to the method of Sharif *et al.* (1989). In addition, the medium was buffered with 20mM Hepes buffer and contained 0.10 g sodium pyruvate/l.

Animals

Sexually mature cross-bred ewes were maintained on open pasture and checked twice a day for oestrus with a vasectomized ram. On Day 8 or 9 of the oestrous cycle, the animals were placed in individual pens in a confinement facility for the duration of the experiment.

Endometrial explant cultures

Ewes were anaesthetized with xylazine/ketamine and maintained with halothane on closed-system anaesthesia. Uteri were exposed via midventral laparotomy. Uterine horns were flushed with 25 ml Dulbecco's phosphate-buffered saline. Ewes were then hysterectomized and the uterus was placed on ice prior to further processing. Endometrium was stripped from underlying tissues from the tip of each horn to the centre of the uterine body. The endometrium was sectioned into small slices (2–3 mm) and rinsed twice with culture medium prior to placement into culture.

Individual cultures of 500 mg wet tissue weight in 10 ml culture medium were placed in 100 $\times$ 20 mm Petri dishes. Culture medium was supplemented with 250 μ Ci of L-[3 H]leucine. Tissue samples were cultured for 24 h at 37°C in an atmosphere of 55% N₂, 45% O₂ and 5% CO₂ by volume, on a rocking platform. Cultures were terminated by immediate placement on ice, followed by centrifugation at 3000 g for 20 min at 4°C. Supernatant solutions were extensively dialysed against distilled water containing 0.1mM phenylmethane sulphonyl fluoride and 0.02% NaN₃. Cultures were frozen at –20°C until processed.

Protein analysis

Secreted proteins were evaluated by two-dimensional electrophoresis as follows: aliquots of culture medium containing approximately 8 $\times$ 10⁵ d.p.m. (nondialysable counts) were dried by means of a Savant Speed-Vac and

Table 1. Effect of intramuscular injection of recombinant bovine interferon- α_1 (rboIFN- α_1) on the subsequent ability of ewe endometrial explants to incorporate L-[3 H]leucine into proteins released into the culture medium

Treatment	Source of endometrial tissue	Protein in medium* (mg/ml)	Radioactivity incorporated* (d.p.m. $\times 10^{-7}$)
Vehicle	Pooled tissue from both uterine horns	0.74 $\pm$ 0.02 (4)	1.89 $\pm$ 0.35 (5)
rboIFN- α_1	Ipsilateral horn	0.93 $\pm$ 0.02 (4)	1.95 $\pm$ 0.39 (4)
rboIFN- α_1	Contralateral horn	0.91 $\pm$ 0.01 (4)	1.61 $\pm$ 0.30 (4)

Endometrial explants were cultured, as described in the Methods, from Day 13 oestrus ewes injected with either vehicle ($n = 5$) or rboIFN- α_1 .

*Data are average values $\pm$ s.e.m. (with numbers of ewes in parentheses). No significant differences were observed between treatments or between tissues from the uterine horns ipsi- or contralateral to the corpus luteum ($P > 0.1$). None of the ewes used had bilateral corpora lutea.

reconstituted in 55 μ l solubilization buffer for isoelectric focusing in the first dimension. After second-dimension electrophoretic separation on a 12.5% polyacrylamide gel containing sodium dodecyl sulphate (Roberts *et al.*, 1984), the proteins were stained with Coomassie Brilliant Blue R-250 and treated with Enhance (New England Nuclear, Boston, MA, USA) prior to fluorography. Fluorographs were prepared on Kodak XAR-5 radiographic film and exposed for 13 days.

Experiment 1

Cyclic ewes were injected with either 2 mg rboIFN- α_1 ($n = 5$) or vehicle alone (control; $n = 4$) at 12-h intervals beginning on Day 11 of the oestrous cycle and ending on the morning of Day 13, i.e. five injections per ewe. Hysterectomies were performed ~ 8 h after the last injection. Explant cultures were prepared in triplicate (500 mg each). Cultures were prepared from each uterine horn of ewes treated with rboIFN- α_1 and evaluated separately. Tissue from control ewes was pooled for evaluation. Secreted proteins were analysed by two-dimensional electrophoresis.

Experiment 2

Endometrium from cyclic Day 13 ewes ($n = 4$; Expt 1 controls) was partitioned into five treatment groups: control, 20 ng/ml or 200 ng/ml rboIFN- α_1 or 20 or 200 ng/ml rbTP-1 added per culture. Triplicate cultures were prepared for each treatment group and cultured as indicated. Proteins in culture medium were analysed as in Expt 1.

Statistical procedures

The data for incorporation of L-[3 H]leucine into nondialysable materials in the culture medium were evaluated by analysis of variance with treatment, treatment dose and tissue source (contralateral vs. ipsilateral uterine horn) (SAS, 1985).

Results

Experiment 1

Endometrial explants from ewes injected with rboIFN- α_1 incorporated similar amounts of L-[3 H]leucine into nondiffusible products as ewes receiving vehicle (controls) (Table 1). No significant differences were observed between tissues obtained from the uterine horns ipsilateral or contralateral to the ovary bearing the corpus luteum ($P > 0.1$). The medium from these cultures contained similar amounts of protein. Most of this protein can probably be accounted for as non-radioactive serum components that leached out of the tissue during incubation. For example, the large clear area noted in the upper central areas of the fluorographs in Figs 1 and 2 represents

Fig. 1. Comparison of polypeptide production of radioactive polypeptides released by cultured endometrial explants derived from Day 13 cyclic ewes receiving vehicle (control) or recombinant bovine interferon- α_1 (rboIFN- α_1) i.m. Plates are representative fluorographs showing two-dimensional polyacrylamide gels loaded with equivalent amounts of radioactivity and exposed to X-ray film for the same period: (a) from a control animal, (b) from the ipsilateral and (c) contralateral horns of a ewe injected i.m. with rboIFN- α_1 . The p70 protein is enhanced in both horns, as indicated by arrows.

unlabelled serum albumin. Its presence, and that of other less abundant serum proteins, invariably causes some distortion of the pattern of labelled products on two-dimensional gels.

The p70 protein was clearly evident in all cultures derived from ewes that had been injected with rboIFN- α_1 (Fig. 1). It was invariably absent in the control (vehicle-injected) group. The amount of

Fig. 2. Comparison of polypeptide production by cultured explants derived from control Day 13 ewe endometrium incubated with (a) 20 or (b) 200 ng/ml recombinant bovine interferon- α_1 (rboIFN- α_1); (c) 20 or (d) 200 ng/ml recombinant bovine trophoblast protein-1 (rbTBP-1). Plates are representative fluorographs of two-dimensional polyacrylamide gels. The arrows indicate location of the p70 protein, which is apparent in all four cultures.

p70 produced was always greater in the explants from IFN-injected ewes than in cultures from placebo ewes where the explants had been provided with rboIFN- α_1 only for the 24-h culture period (Fig. 2).

Experiment 2

Endometrial explants from Day 13 cyclic ewes incorporated $1.89 \pm 0.35 \times 10^7$ d.p.m. of radio-label from L-[^3H]leucine into nondiffusible components in the culture medium during a 24-h period (Table 2). This control value was not significantly different from values noted for tissues that had been cultured in the presence of either rbTBP-1 or rboIFN- α_1 ($P > 0.1$). Endometrial tissue from a Day 13 cyclic ewe only produced increased amounts of p70 if it had been cultured in presence of either bTBP-1 or rboIFN- α_1 (Fig. 2). Both kinds of IFN were equally effective in inducing p70 when they were supplied at 200 ng/ml ($\sim 10\text{nM}$) (Fig. 2). At the lower concentration of IFN (20 ng/ml; 1nM), p70 appeared to be induced less effectively in cultures supplied with rbTBP-1 than with rboIFN- α_1 , compared with control cultures (Fig. 2a).

Table 2. Effects of interferons recombinant bovine trophoblast protein-1 (rbTP-1) and recombinant bovine interferon- α_1 1 (rboIFN- α_1 1) on incorporation of L-[3 H]leucine into proteins released into the medium by cultured endometrial explants

Treatment	Concentration of IFN added to medium (ng/ml)	Protein in medium* (mg/ml)	Radioactivity incorporated* (d.p.m. $\times 10^{-7}$)
Control	—	0.74 $\pm$ 0.02 (5)	1.89 $\pm$ 0.35 (5)
rbTP-1	20	0.68 $\pm$ 0.05 (4)	2.07 $\pm$ 0.20 (4)
rbTP-1	200	0.67 $\pm$ 0.05 (4)	2.25 $\pm$ 0.37 (4)
rboIFN- α_1 1	20	0.69 $\pm$ 0.03 (4)	1.70 $\pm$ 0.46 (4)
rboIFN- α_1 1	200	0.69 $\pm$ 0.09 (4)	1.51 $\pm$ 0.42 (4)

Endometrial explants were cultured as described in the Methods. Explant tissues were derived from the vehicle-injected ewes also used for data in Table 1.

*Data are average values $\pm$ s.e.m. (with numbers of ewes in parentheses). No significant differences ($P > 0.1$) were observed between treatments. The ewes used in this study were at Day 13 of their oestrous cycles.

Discussion

These experiments confirm that a recombinant α_1 interferon (rboIFN- α_1 1) and the longer (172 residue vs. 166) recombinant bTP-1 can, like oTP-1 (Salamonsen *et al.*, 1988; Silcox *et al.*, 1988), induce p70 synthesis by endometrial tissue derived from ewes in the mid-luteal phase of their oestrous cycles. The effects of rbTP-1 and rboIFN- α_1 1 were comparable when they were supplied *in vitro* at 10nM, but the latter seemed more potent at 1nM. One explanation is that the binding constants of rbTP-1 and rboIFN- α_1 1 for the Type 1 IFN receptor, which is abundant in endometrium (Hansen *et al.*, 1989; Knickerbocker & Niswender, 1989), differ. Alternatively, even though the rbTP-1 and rboIFN- α_1 1 had comparable antiviral activity initially (Klemann *et al.*, 1990), the rbTP-1 may be less stable and hence less effective over the 24-h culture period. Whatever the explanation, it is evident from this and experiments performed earlier that a range of different IFN- α can induce p70 synthesis and that this protein provides a sensitive and useful marker for IFN action on uterine endometrial tissue (Silcox *et al.*, 1988, Sharif *et al.*, 1989). It remains to be determined whether this protein plays any role in maternal recognition of pregnancy in ewes. It is also unclear whether it is secreted towards the uterine lumen *in vivo*.

Expt 1 demonstrated that when rboIFN- α_1 1 was injected into ewes at a comparable time after oestrus and at doses identical to those shown to improve pregnancy rates in bred ewes (Nephew *et al.*, 1990; Schalue-Francis *et al.*, 1991), there was a pronounced effect on the synthesis of p70 by the endometrium of both uterine horns. Therefore, it would not be unreasonable to expect other aspects of uterine biochemical activity, such as prostaglandin metabolism (Salamonsen, 1988; Helmer *et al.*, 1989), oxytocin receptor synthesis (Bazer *et al.*, 1991) or turnover of second messengers (Mirando *et al.*, 1990) to be similarly affected throughout the endometrium. The antiviral activity of rboIFN- α_1 1 in the blood of ewes following a 2.0-mg injection rises to a maximum of ~ 500 International Reference Units (IRU) within 2 h and then falls to ~ 200 units/ml prior to the next injection 12 h later (Schalue-Francis *et al.*, 1991). Since the IFN had an activity of $\sim 5 \times 10^7$ IRU/mg the concentration of active IFN in the circulation would range between about 0.5nM and 0.2nM during the 2½-day experiment. Although these concentrations are low, they are still above the K_D of the rboIFN- α_1 1 for the Type 1 ovine IFN receptor, which is probably $\sim 10^{-11}$ M (Hansen *et al.*, 1989; Knickerbocker & Niswender, 1989). The amount of p70 produced by these cultures from injected ewes appeared greater than that from the explants which had only been exposed to IFN in culture, and was comparable to p70 production observed with tissue from pregnant ewes (Sharif *et al.*, 1989). This difference may relate to the time required for full induction of p70 *in vitro*. The effect was noted in both uterine horns, whereas in pregnancy the oTP-1

produced by the conceptus has a local action confined to regions of endometrium in contact with trophoblast (Sharif *et al.*, 1989).

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