Onset of secretion of proteins with antiviral activity by pig conceptuses*

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Summary. In Exp. 1, antiviral activity was detected in Day-15 pregnant uterine flushings (6222 ± 2167 units/ml) and in conceptus culture medium collected at 0, 1, 2, 4, 8, 16, 24, 32, 40, and 48 h (95, 375, 650, 1216, 1600, 2100, 2017, 2083, 3500 and 5000 units/ml, respectively; $R^2 = 0.81$, $P < 0.01$; $y = 190.0 + 252.7x - 11.2x^2 + 0.2x^3$). In Exp. 2, antiviral activity of Day-15 conceptus culture medium was reduced 99% after boiling for 20 min ($P < 0.01$) and, after 18 h dialysis (6000–8000 M_r cut-off), 100% of the activity was in the retentate. In Exp. 3, antiviral activity was not detected in cultures of conceptuses from Days 10 and 11 and activity was maximal for Day 14 and Day 15 conceptuses (2100 and 2083 units/ml, respectively). Effects of day were best described by a quadratic regression equation ($y = 17\,652 - 3263x + 150x^2$; $R^2 = 0.55$, $P < 0.01$). In Exp. 4, changes in antiviral activity detected in uterine flushings from pregnant gilts on Days 8, 10, 11, 12, 14 and 15 (1.3, 0, 6.7, 63.3, 580 and 1663 units/ml, respectively) were described by the equation $y = -20\,743 + 6189x - 606x^2 + 20x^3$ ($R^2 = 0.85$, $P < 0.01$). In Exp. 5, low antiviral activities (5–30 units/ml) were detected in all plasma samples collected from the uterine artery and uterine vein of pregnant and cyclic gilts, but values were not significantly influenced by pregnancy status, day or site of collection.

These results indicate that proteins with antiviral activity are secreted by pig conceptuses after Day 10 of pregnancy with highest activities on Days 14 and 15. The presence of conceptuses does not influence antiviral activity in the uterine vasculature.

Keywords: pig; pregnancy; conceptus; protein; interferon

Introduction

Proteins secreted by elongating conceptuses of sheep and cattle (ovine trophoblast protein-1 and bovine trophoblast protein-1, respectively) are involved in establishment of pregnancy (Bazer *et al.*, 1987; Helmer *et al.*, 1988; Vallet *et al.*, 1988). These proteins have a high degree of amino acid sequence homology with several known α -interferons (Imakawa *et al.*, 1987, 1989; Stewart *et al.*, 1987; Charpigny *et al.*, 1988) and oTP-1 possesses potent antiviral activity *in vitro* (Pontzer *et al.*, 1988). In pigs, oestradiol produced by conceptuses on Days 10–12 is the primary signal for establishment of pregnancy (Bazer *et al.*, 1982). However, elongating pig conceptuses also secrete low molecular weight (M_r), acidic proteins (Godkin *et al.*, 1982), some of which have been reported to cross-react with antiserum to human α -interferon and possess antiviral activity (Cross & Roberts, 1988).

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These experiments were conducted to: (1) demonstrate that pig conceptuses secrete protein(s) with antiviral activity; (2) determine time of onset of secretion of proteins with antiviral activity *in vitro* and *in vivo*; and (3) determine whether proteins with antiviral activity are released from the uterus into the uterine circulation.

Materials and Methods

Animals. Sexually mature crossbred (Duroc × Yorkshire × Hampshire) gilts were observed daily at 07:30 h for oestrous behaviour in the presence of an intact boar. Gilts assigned to the pregnant status were mated when detected in oestrus (Day 0) and at 10 and 24 h after onset of oestrus. All gilts were laparotomized while under general anaesthesia, induced with 1 g thiamylal sodium (*i.v.*) and maintained with 3–5% halothane, and the reproductive tract removed.

Experiment 1. Three pregnant gilts were hysterectomized on Day 15. Each uterine horn was flushed with 20 ml modified Eagle's Minimum Essential Medium (MEM) and 500 mg conceptus tissue were removed, placed in 15 ml MEM and cultured as described by Godkin *et al.* (1982). The remaining uterine flush was removed and centrifuged at 1800 *g* and 10°C for 4 min to remove tissue debris. The supernatant was decanted and stored at –20°C until assayed for antiviral activity. Samples of medium (250 µl) from conceptus cultures were obtained at 0, 1, 2, 4, 8, 16, 24, 32, 40 and 48 h after addition of conceptus tissue and stored at –20°C until assayed for antiviral activity. Medium removed during sampling was not replaced with fresh medium.

Experiment 2. Three pregnant gilts were hysterectomized on Day 15 and 500 mg conceptus tissue were cultured in 15 ml MEM for 30 h. After 30 h, medium was removed, centrifuged at 1800 *g* and 4°C for 10 min and the supernatant decanted. An aliquant of supernatant was removed for the antiviral assay. A second aliquant (0.5 ml) was removed and placed in a boiling water bath for 20 min before the antiviral assay. A third aliquant (5.0 ml) was removed and dialysed (M_r 6000–8000 cut-off membrane: Spectrum Medical Industries, Inc., Los Angeles, CA, USA) against 30 ml 10 mM-sodium acetate and 1 mM-EDTA for 18 h. Aliquants of dialysate and retentate were removed at 0 and 18 h after dialysis and assayed for antiviral activity.

Experiment 3. Pregnant gilts were hysterectomized on Days 10 (*N* = 3), 11 (*N* = 3), 12 (*N* = 6), 13 (*N* = 3), 14 (*N* = 3) and 15 (*N* = 6) and each uterine horn was flushed with 20 ml sterile MEM. The conceptuses were removed and cultured (200 mg tissue/15 ml MEM) for 30 h. When 200 mg conceptus tissue was not obtained from gilts on Days 10, 11 or 12, the volume of MEM used was reduced such that tissue was cultured at a constant rate of 13.3 mg/ml. After 30 h of culture, medium was removed, centrifuged at 1800 *g* and 4°C for 10 min and the supernatant from each culture stored separately at –20°C until assayed for antiviral activity.

Experiment 4. Gilts were hysterectomized on Days 8 (*N* = 3), 10 (*N* = 3), 11 (*N* = 3), 12 (*N* = 5), 14 (*N* = 3) and 15 (*N* = 4) of pregnancy and Days 10 (*N* = 3) and 14 (*N* = 3) of the oestrous cycle. Each uterine horn was flushed with 20 ml sterile 0.9% (w/v) NaCl or MEM to collect conceptuses and confirm pregnancy. After conceptus tissue was removed, the uterine flushing was centrifuged and the supernatant stored at –20°C until assayed. Only 3 uterine flushings utilized in this experiment were obtained from gilts in Exp. 3.

Experiment 5. Blood (5 ml) was collected from the uterine artery and uterine vein of pregnant gilts on Days 12 (*N* = 3) and 15 (*N* = 3) and cyclic gilts on Day 15 (*N* = 3). The blood was immediately added to tubes containing heparin, placed on ice and then centrifuged for 10 min at 1800 *g* and 4°C. Plasma was harvested and stored at –20°C until assayed for antiviral activity.

Antiviral activity. Samples were assayed for antiviral activity using a cytopathic effect assay (Familletti *et al.*, 1981; Pontzer *et al.*, 1988). Briefly, dilutions of samples were incubated with Madin–Darby bovine kidney (MDBK) cells for 22–24 h at 37°C. Following incubation, inhibition of replication of vesicular stomatitis virus was determined. One antiviral unit prevented 50% destruction of the MDBK cell monolayer. Sensitivity of the assay was <3 antiviral units/ml.

Statistical analysis. Data were analysed by the General Linear Models (GLM) procedure of the Statistical Analysis System (SAS, 1982). For Exps 1, 3 and 4, data were subjected to regression analysis to determine effects of hour or Day. In Exp. 2, data were tested for effects of boiling by one-way analysis of variance (ANOVA). Differences in antiviral activities of dialysate and retentate were tested by one-way ANOVA. In Exp. 5, data were analysed by two-way ANOVA with gilt nested within group (*i.e.* Day 12 pregnant, Day 15 pregnant and Day 15 cyclic).

Results

Experiment 1

Antiviral activity in medium from Day-15 pig conceptus cultures at onset of culture (0 h) was approximately 1.5% of that present in uterine flushings from which the conceptuses were recovered

(95 ± 54 vs 6333 ± 2167 antiviral units/ml, respectively). Regression of antiviral activity on duration of culture indicated that changes were best described by the equation $y = 190.0 + 252.7x - 11.2x^2 + 0.2x^3$ ($R^2 = 0.80$, $P < 0.01$). Antiviral activity increased during the first 16 h of culture, remained constant during the next 16 h and increased again during the final 16 h of culture (Fig. 1).

Fig. 1. Secretion of proteins with antiviral activity (●; mean $\pm$ s.e.m.) into culture medium by Day-15 pig conceptuses (Exp. 1). Conceptus tissue (500 mg) from 3 gilts was cultured in 15 ml MEM for 48 h and samples collected (250 μ l) at 0, 1, 2, 4, 8, 16, 24, 32, 40 and 48 h. Regression of antiviral activity on duration of culture indicated that changes were best described by the equation $y = 190.0 + 252.7x - 11.2x^2 + 0.2x^3$ (—, $R^2 = 0.80$, $P < 0.01$).

Experiment 2

Antiviral activity in medium from Day-15 pig conceptus cultures was reduced ($P < 0.01$) by more than 99% after boiling for 20 min (Table 1), and all activity was in the retentate following dialysis (M_r 6000–8000 cut-off) compared to antiviral activity in medium collected before dialysis (Table 1).

Table 1. Effects of dialysis and boiling on antiviral activity (units/ml) of proteins secreted by Day-15 pig conceptuses in culture (Exp. 2)

	Predialysis/ preboiling	After dialysis	After boiling
Culture medium	767 ± 233	767 ± 233	$8 \pm 4^*$
Dialysate	<3	<3	—

*Antiviral activity (units/ml) was reduced ($P < 0.01$) after boiling.

Experiment 3

Antiviral activity was not detectable in culture medium from Day-10 and Day-11 pig conceptuses and very low activity (10 units/ml) was present in only 1 of 6 cultures with Day-12 conceptuses

(Fig. 2). Antiviral activity was detected in MEM from all cultures of Day-13 (34.7 ± 32.7 units/ml) conceptuses and increased over 60-fold in cultures of Day-14 (2100 ± 1069 units/ml) and Day-15 (2083 ± 682 units/ml) conceptuses. These changes in activity due to day of pregnancy were best described by the equation $y = 17\,652 - 3263x + 150x^2$ ($R^2 = 0.55$, $P < 0.01$).

Fig. 2. Secretion of proteins with antiviral activity (●; mean $\pm$ s.e.m.) into culture medium by Day 10 ($N = 3$), 11 ($N = 3$), 12 ($N = 6$), 13 ($N = 3$), 14 ($N = 3$) and 15 ($N = 6$) pig conceptuses (Exp. 3). Conceptus tissue (13.3 mg/ml MEM) was cultured for 30 h. Regression of antiviral activity on day of pregnancy indicated that changes were best described by the equation $y = 17\,652 - 3263x + 150x^2$ (—, $R^2 = 0.55$, $P < 0.01$).

Experiment 4

Very low antiviral activity was detected in uterine flushings collected from 2 of 3 gilts on Day 8 (2 units/ml) and 1 of 3 gilts on Day 10 (20 units/ml) of pregnancy, but no activity was detected in uterine flushings from gilts on Day 11 of pregnancy (Fig. 3). By Day 12 of pregnancy, antiviral activity was detectable in all uterine flushings (63.3 ± 22.5 units/ml) and increased through Day 15 of pregnancy as described by the equation $y = -20\,743 + 6189x - 606x^2 + 20x^3$ ($R^2 = 0.85$, $P < 0.01$). Uterine flushings from cyclic gilts on Days 10 and 14 had no detectable antiviral activity.

Experiment 5

Low antiviral activity (< 30 units/ml) was detected in all plasma samples collected from the uterine artery and uterine vein of pregnant and cyclic gilts. Mean antiviral activity in uterine vein and uterine artery of gilts was 10.0 and 7.2, 17.2 and 16.7, and 15.5 and 16.7, respectively, on Days 12 and 15 of pregnancy and Day 15 of the oestrous cycle (s.e.m. = 1.73). However, amount of activity was not significantly influenced by source of plasma (artery versus vein), pregnancy status or day.

Discussion

The results of the present study indicate that pig conceptuses secrete proteins possessing antiviral activity and support results reported by Cross & Roberts (1988). Low antiviral activity was detected

Fig. 3. Antiviral activity (●; mean $\pm$ s.e.m.) of proteins in uterine flushings from pregnant gilts on Days 8 (N = 3), 10 (N = 3), 11 (N = 3), 12 (N = 5), 14 (N = 3) and 15 (N = 4) (Exp. 4). Each uterine horn was flushed with 20 ml MEM. Regression of antiviral activity on day of pregnancy indicated that changes were best described by the equation $y = -20\,743 + 6189x - 606x^2 + 20x^3$ (—, $R^2 = 0.85$, $P < 0.01$).

in medium immediately (0 h) after addition of conceptuses. This was probably due to introduction of antiviral protein(s) in uterine flushings absorbed to conceptus tissue rather than rapid and immediate secretion of antiviral protein(s) by conceptuses. Secretion of antiviral proteins was rapid and their activity in culture medium increased almost 4-fold during the first hour of culture. Secretion of antiviral proteins continued throughout the 48-h culture period; increasing from 0 to 16 h, remaining constant from 16 to 32 h and increasing again from 40 to 48 h. This was unexpected because incorporation of [¹⁴C]leucine into macromolecules secreted during the first 24 h of culture by pig conceptuses was linear (Godkin *et al.*, 1982). Failure of antiviral activity to increase during the second 16 h of culture suggests that secretion of antiviral proteins ceased or that secretion and degradation of the antiviral proteins occurred at similar rates during that period. The latter seems more likely since antiviral activity was increased by the addition of a protease inhibitor to conceptus culture medium (Beers *et al.*, 1990). Evidence exists for stimulatory and inhibitory actions of the uterus on secretion of proteins by pig conceptuses (Rice *et al.*, 1981; Beers *et al.*, 1990). Specifically, secretion of antiviral proteins by conceptus tissue *in vitro* was reduced in co-cultures with Day-15 pregnant endometrium or medium enriched in Day-14 cyclic endometrial secretions (Beers *et al.*, 1990).

Antiviral activity was very low or undetectable in uterine flushings and conceptus culture medium representing Days 8–13 of pregnancy. When activity was present on those days, it was generally greater in uterine flushings. These results suggest that conceptuses from these days produce more antiviral proteins *in vivo* than *in vitro*. The possibility of a regulatory role for the uterine endometrium upon secretion of antiviral proteins also exists. Ashworth & Bazer (1989) reported that secretion of oTP-1 by ovine conceptuses was stimulated during co-culture with endometrium from Day-16 pregnant ewes. Secretory products of pig endometrium are also capable of stimulating protein secretion by pig conceptuses (Rice *et al.*, 1981), as well as inhibiting secretion of antiviral proteins (Beers *et al.*, 1990).

Secretion of antiviral proteins by conceptuses collected and cultured before Day 12 of pregnancy was not detected and only 1 of 6 cultures of Day-12 conceptuses had detectable activity. This is in contrast to the report of Cross & Roberts (1988) that 1000 antiviral units/conceptus were secreted *in vitro* by pig conceptuses collected on Day 11 of pregnancy. The reason for this difference is not clear. Both laboratories utilized similar culture techniques (Godkin *et al.*, 1982) and antiviral assays. Pig interferon- α was reported to lose activity during storage (Piasecki, 1988) and pig

conceptuses secreted a group of low molecular-weight acidic proteins which cross-reacted with anti-human α -interferon and possessed antiviral activity (Cross & Roberts, 1988). However, we have not detected significant loss of antiviral activity from conceptus culture medium during storage at -20°C for 5–7 months (M. A. Mirando, J. P. Harney & F. W. Bazer, unpublished observations). Moreover, antiviral activity in pig conceptus culture medium was not reduced after freezing and thawing and, when medium was assayed unfrozen immediately after culture, antiviral activity was detected in medium of conceptuses from only 1 of 3 Day-12 pregnant gilts (Beers *et al.*, 1990).

All samples of blood plasma collected from the uterine artery and uterine vein of pregnant and cyclic gilts on Days 12 and 15 after oestrus had similar antiviral activities. This indicated that antiviral activity in uterine flushings of pregnant gilts is neither secreted into nor acquired from the peripheral circulation. Secretion of oTP-1 into the uterine vein of pregnant ewes has not been detected (Pontzer *et al.*, 1988). The presence of antiviral activity in blood, but not uterine flushings, from cyclic gilts provides further evidence that the uterus does not actively remove antiviral proteins from the circulation and that these proteins are pregnancy (conceptus)-specific products.

Secretion *in vivo* and *in vitro* of antiviral proteins by pig conceptuses during the period of establishment of pregnancy (i.e. Days 10–12) was very low and did not increase appreciably until Days 14 and 15, several days after the antiluteolytic oestrogen signal originates from the conceptus (Bazer *et al.*, 1982). Intrauterine administration of pig conceptus secretory proteins to gilts did not lengthen interoestrous interval (Harney & Bazer, 1989), whereas intrauterine infusion of bovine and ovine conceptus secretory proteins increased the interoestrous intervals of cows and ewes, respectively (Knickerbocker *et al.*, 1986; Vallet *et al.*, 1988). Further, secretion of prostaglandins F-2 α and E-2 was stimulated by administration of pig conceptus secretory proteins *in vivo* (Harney & Bazer, 1989) and *in vitro* (Dubois & Bazer, 1988). Therefore, it is unlikely that the antiviral protein(s) secreted by the conceptuses, as described in the present study and by Cross & Roberts (1988), are involved in extending CL lifespan in pigs.

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