Influence of the endometrium, protease inhibitors and freezing on antiviral activity of proteins secreted by pig conceptuses*

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Summary. In Exp. 1, only medium from cultures containing conceptus tissue had antiviral activity ($P < 0.05$). Addition of Day-15 pregnant endometrium or Day-14 cyclic uterine flush proteins to cultures containing 200 mg conceptus tissue decreased antiviral activity (conceptus × endometrial protein interaction, $P < 0.06$). Effects of endometrium ($-54\%$) and uterine flush proteins ($-40\%$) on antiviral activity of conceptus cultures did not differ from each other ($P > 0.10$). In Exp. 2, antiviral activity was only detected in cultures containing conceptus tissue ($P < 0.06$). The amount of antiviral activity in cultures of Day-15 conceptus tissue was not influenced differently ($P > 0.10$) by culture in medium conditioned by endometrium from Day 10 or Day 12 of pregnancy. However, antiviral activity was undetectable in medium conditioned by endometrium from one of the Day-12 gilts. In Exp. 3, antiviral activity was present in medium from only 1 of 3 cultures from Day-12 gilts when assayed unfrozen. Antiviral activity was lower ($P < 0.01$) in cultures of conceptuses from Day 12 than Day 14 of pregnancy; however, antiviral activity increased quadratically ($P < 0.05$) when cultures contained 0, 0-01, 0-1 and 1-0 units/ml aprotinin, respectively. Freezing and thawing culture medium did not reduce ($P > 0.10$) antiviral activity compared to medium assayed unfrozen (1438 vs 1354 units/ml, respectively). These results suggest a regulatory influence of the endometrium on secretion of antiviral proteins by pig conceptuses in vitro.

Keywords: pig; pregnancy; conceptus; interferon; endometrium; proteolysis

Introduction

Proteins secreted by elongating conceptuses of sheep (ovine trophoblast protein-1, oTP-1) and cattle (bovine trophoblast protein-1, bTP-1) are involved in inhibition of luteolysis and establishment of pregnancy (Godkin et al., 1984a; Knickerbocker et al., 1986; Helmer et al., 1988; Vallet et al., 1988). These proteins have high amino acid sequence homology with alpha II interferons (Imakawa et al., 1987, 1987; Stewart et al., 1987; Charpigny et al., 1988) and oTP-1 has potent antiviral activity (Pontzer et al., 1988). Elongating pig conceptuses also secrete a group of low molecular weight acidic proteins (Godkin et al., 1982). These proteins have been reported to be secreted as early as Day 11 of pregnancy, possess antiviral activity in vitro and cross-react with antiserum to human a-interferon (Cross & Roberts, 1988). In contrast to the antiluteolytic effects of oTP-1 and bTP-1, pig conceptus secretory proteins do not inhibit luteolysis when infused into the uterine lumen of cyclic gilts (Harney & Bazer, 1989). Rather, they stimulate endometrial

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secretion of PGF-2α and PGE-2 in vivo (Harney & Bazer, 1989) and in vitro (Dubois & Bazer, 1988). Oestrogens from pig conceptuses appear to be responsible for establishment of pregnancy in pigs (Bazer et al., 1982).

Secretion of several proteins by the sheep endometrium is stimulated by addition of oTP-1 to culture medium (Godkin et al., 1984b; Vallet et al., 1987; Ashworth & Bazer, 1989). Conversely, oTP-1 secretion by Day-16 conceptuses is stimulated by co-culture with endometrium from ewes on Day 16 of pregnancy (Ashworth & Bazer, 1989). Secretory products of pig endometrium also stimulate protein secretion by pig blastocysts (Rice et al., 1981). This study determined the influence of the endometrium, inhibition of proteolysis and freezing on antiviral activity of proteins secreted by pig conceptuses in vitro.

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. Four pregnant gilts were hysterectomized on Day 15. Each uterine horn was flushed with 20 ml sterile 0-9% (w/v) NaCl to collect conceptuses and confirm pregnancy. Endometrium was dissected from the underlying myometrium and placed in modified Eagle’s Minimum Essential Medium (MEM) as described by Godkin et al. (1982). Conceptus tissue (0 or 200 mg) was cultured in a 2 × 3 factorial arrangement in the presence of medium only, 200 mg endometrium or 75 µg uterine flush proteins collected from a Day 14 cyclic gilt as described by Bazer et al. (1978).

The uterine flush proteins were prepared by concentrating 10 ml of uterine flushing to approximately 3 ml with an Amicon Centriprep device (W. R. Grace & Co., Danvers, MA, USA) by centrifugation for 40 min at 4°C and 4000 g. The concentrated proteins were then centrifuged for 4 min at 4°C and 13 000 g to remove particulate matter. Protein concentration was determined by the method of Lowry et al. (1951) to be 5 mg/ml.

All combinations of conceptus, endometrium and uterine flush proteins were cultured for 30 h in 15 ml MEM (Godkin et al., 1982); medium was then removed, centrifuged to remove cell debris and the supernatant stored at −20°C until assayed for antiviral activity.

Experiment 2. Six pregnant gilts were hysterectomized on Days 10 and 12 (3 gilts/day). The reproductive tracts were removed and endometrium was dissected from the underlying myometrium. Endometrium (200 mg) was cultured in 15 ml MEM and, after 30 h, medium was removed, centrifuged to remove cell debris, and the supernatant stored at −20°C until thawed for use as conceptus culture medium.

Conceptuses were collected from 1 pregnant gilt on Day 15 by flushing the uterine horns with 20 ml sterile MEM. Conceptus tissue (100 mg) was cultured for 30 h in 7.5 ml of medium conditioned by culture of endometrium from Days 10 and 12 of pregnancy. Conceptus culture medium was centrifuged to remove cell debris, and the supernatant stored at −20°C.

Experiment 3. Six pregnant gilts were hysterectomized on Days 12 and 14 (3 gilts/day). Each uterine horn was flushed with 20 ml sterile MEM and conceptus tissue cultured (33.3 mg/ml) in the presence of 0, 0.01, 0.1 and 1.0 trypsin inhibitory units/ml of aprotinin (Sigma Chemical Co., St Louis, MO, USA). After 30 h, culture medium was removed, centrifuged at 1800 g and 4°C for 10 min and the supernatant recovered. An aliquant of the supernatant was removed and frozen at −20°C for approximately 1.5 h before thawing at room temperature. The remaining culture medium was stored at 4°C for approximately 1.5 h before warming to room temperature. The antiviral activity in all samples of culture medium were quantified within 2 h of removal from culture.

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

Statistical analysis. Data were subjected to analysis of variance (ANOVA) using the General Linear Models (GLM) procedure of the Statistical Analysis System (SAS, 1982). For Exp. 1, data were analysed by two-way ANOVA for 2 × 3 factorial arrangement of treatments. For Exp. 2, data were analysed by two-way ANOVA for 2 × 2 factorial arrangement of treatments with gilts nested within day of pregnancy. For Exp. 3, data were transformed to log10 and analysed by three-way ANOVA for 2 × 2 × 4 factorial arrangement of treatments with gilts nested within day of pregnancy. Tests of hypotheses were performed using appropriate error terms according to the mean-squares expected (Snedecor & Cochran, 1980).
Results

Experiment 1

Culture medium of conceptuses from one gilt did not possess antiviral activity; therefore, data from all treatment combinations for that gilt were excluded from the analysis. Significant antiviral activity was present only in culture medium containing conceptus tissue \( (P < 0.05, \text{Table 1}) \). Conceptuses cultured with Day-15 pregnant endometrium \((-54\%)\) or Day-14 cyclic uterine flush proteins \((-40\%)\) had reduced \( (P < 0.05) \) secretion of antiviral proteins into medium. However, the interaction of conceptus tissue with uterine protein \( (P < 0.06) \) indicated that inhibitory effects of endometrium and uterine flush proteins occurred primarily in cultures containing 200 mg conceptus tissue.

<table>
<thead>
<tr>
<th>Co-culture treatment</th>
<th>Conceptus tissue (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Medium only</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Endometrium (200 mg)</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Uterine flushing proteins (75 µg)</td>
<td>&lt;3</td>
</tr>
</tbody>
</table>

0 mg vs 200 mg conceptus tissue, \( P < 0.05 \).
Medium vs endometrium + uterine flush proteins, \( P < 0.05 \).
Conceptus \( \times \) co-culture treatment interaction, \( P < 0.06 \).
Standard error of least-square means = 132.6.

Experiment 2

No antiviral activity was detected in endometrium-conditioned culture medium, but was present in that medium after culture of conceptuses \( (P < 0.05, \text{Table 2}) \). Secretion of antiviral proteins by conceptus tissue was not influenced differently \( (P > 0.10) \) by culture medium conditioned by endometrium collected on Day 10 compared to Day 12 of pregnancy. However, antiviral activity from conceptus tissue was inhibited completely when cultured in medium conditioned by endometrium from 1 gilt on Day 12 of pregnancy.

Experiment 3

Conceptuses collected from gilts on Day 12 of pregnancy secreted less \( (P < 0.01) \) antiviral activity \emph{in vitro} than those collected on Day 14 (Table 3). For medium assayed unfrozen within 2 h after culture, antiviral activity was detected for only 1 of 3 gilts on Day 12, but all 3 gilts on Day 14. Addition of aprotinin to culture medium of conceptuses increased \( (P < 0.07) \) antiviral activity secreted and orthogonal contrasts detected a quadratic \( (P < 0.05) \) effect. Addition of 0-01 and 0-1 units/ml aprotinin to conceptus cultures increased antiviral activity by 53 and 60%, respectively, while 1-0 unit/ml increased antiviral activity by only 21%. Aprotinin itself did not possess antiviral activity nor did it enhance antiviral activity when added to medium collected after culture of conceptuses (data not shown). Effects of aprotinin on antiviral activity in medium from Day-12
Table 2. Influence of medium conditioned by culture of endometrium from pigs at Days 10 and 12 of pregnancy on antiviral proteins (units/ml) secreted by Day-15 conceptus tissue (Exp. 2)

<table>
<thead>
<tr>
<th>Conditioned culture medium</th>
<th>Conceptus tissue (mg)</th>
<th>0 mg</th>
<th>200 mg</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Mean</td>
<td>Range</td>
<td></td>
</tr>
<tr>
<td>Day 10 endometrium</td>
<td>&lt;3</td>
<td>450</td>
<td>300-750</td>
<td></td>
</tr>
<tr>
<td>Day 12 endometrium</td>
<td>&lt;3</td>
<td>317</td>
<td>&lt;3-650</td>
<td></td>
</tr>
</tbody>
</table>

0 mg vs 200 mg conceptus tissue, $P < 0.05$.
Day 10 vs Day 12 endometrium did not differ, $P > 0.10$.
Standard error of least-squares means = 120.2.

Table 3. The influence of aprotinin and freezing on antiviral proteins (units/ml) secreted in vitro by Day-12 and Day-14 conceptuses (Exp. 3)

<table>
<thead>
<tr>
<th>Day of pregnancy</th>
<th>Condition of medium</th>
<th>Aprotinin conc. (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>Unfrozen</td>
<td>3</td>
</tr>
<tr>
<td>12</td>
<td>Frozen</td>
<td>3</td>
</tr>
<tr>
<td>14</td>
<td>Unfrozen</td>
<td>2600</td>
</tr>
<tr>
<td>14</td>
<td>Frozen</td>
<td>2100</td>
</tr>
</tbody>
</table>

Day 12 vs Day 14, $P < 0.01$.
Aprotinin concentration, $P < 0.07$ (quadratic increase, $P < 0.05$).
Means presented are values for untransformed data and s.e.m = 202. For statistical analysis, data were transformed to log_{10}.

Discussion

The results of the present study indicate that secretory products of pig endometrium can affect secretion of proteins by conceptuses in vitro and agree with the results of Ashworth & Bazer (1989) for sheep and Rice et al. (1981) for pigs. However, the presence of Day-15 pregnant endometrium or Day-14 cyclic uterine flush proteins decreased secretion of antiviral proteins by Day-15 pig conceptuses in vitro. The secretion of oTP-1 by Day-16 sheep conceptuses was enhanced when they were co-cultured with endometrium from Day-16 pregnant ewes (Ashworth & Bazer, 1989). Similarly, Rice et al. (1981) reported that endometrial proteins increased protein synthesis by pig blastocysts. The reasons for these apparent discrepancies are not clear, but differential regulation of individual conceptus secretory proteins by the endometrium, as well as variation among species, may occur. The conceptus can stimulate synthesis of some endometrial secretory proteins while inhibiting synthesis of others in sheep (Vallet et al., 1987; Ashworth & Bazer, 1989). The same may be true for influences of pig endometrium on pig conceptuses.
Antiviral proteins from pig conceptuses

One mechanism for regulating secretion of antiviral protein(s) by conceptuses may include effects of retinoic acid and retinol binding proteins (RBP). Retinoids have been reported to block interferon synthesis in vitro (Blalock & Gifford, 1976, 1977) and pig conceptus secretory proteins with antiviral activity have antigenic similarity to human α-interferon (Cross & Roberts, 1988). Secretion of RBP by pig conceptuses in culture is detectable by Day 12 of pregnancy and increases markedly through Day 15 (Harney & Mirando, 1989). This pattern of RBP secretion is similar to that for secretion of antiviral proteins by pig conceptuses (Mirando et al., 1990). Secretion of another RBP by pig endometrium is influenced by progesterone (Adams et al., 1981). One may speculate that the potentially inhibitory influence of retinoids on secretion of conceptus interferons may be removed by binding of retinoids by RBP. Alternatively, the amount of antiviral activity present may be reduced through enzymic degradation by endometrial proteins secreted.

Antiviral activity in culture medium of conceptuses and uterine flushings from pregnant gilts increased between Days 10 and 15 (Mirando et al., 1990), suggesting that elongated conceptuses have a greater capacity to secrete antiviral proteins and/or endometrium from earlier in pregnancy is more inhibitory to secretion of proteins with antiviral activity. Therefore, it was of interest to examine the relative influence of endometrium obtained at onset of secretion of antiviral protein(s) of Days 10–12. However, antiviral activity from Day-15 conceptuses was not affected differently by endometrium collected on Days 10 and 12 of pregnancy in this study. Endometrial conditioned culture medium representing 1 gilt on Day 12 of pregnancy completely inhibited secretion of antiviral proteins(s) by Day-15 conceptus tissue, but the reason for this is not known. This result supports the concept that secretory products of the endometrium regulate secretion and/or degradation of antiviral proteins from pig conceptuses.

Antiviral activity in conceptus culture medium was not reduced by 1 cycle of freezing and thawing in the present study and loss of antiviral activity of pig conceptus culture medium after repeated freezing and thawing was not detected (M. A. Mirando & F. W. Bazer, unpublished observations). Pig interferon-α was reported to lose activity during storage at −20°C (Piasecki, 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).

Antiviral activity in cultures of conceptuses from Days 12 and 14 of pregnancy was increased by addition of aprotinin to the culture medium. This effect was not due to aprotinin having antiviral activity or enhancing antiviral activity in conceptus culture medium. Rather, aprotinin apparently increased antiviral activity in conceptus culture medium by inhibiting proteolytic destruction of the protein(s) possessing antiviral activity. Pre-attachment pig conceptuses secrete plasminogen activator (Mullins et al., 1980; Fazleabas et al., 1983, 1985) and may produce other proteases as well. This could account for reduced antiviral activity when aprotinin was omitted from conceptus culture medium in the present study. The uterine endometrium also secretes at least one protease inhibitor (Mullins et al., 1980; Fazleabas et al., 1983, 1985). Consequently, removal of conceptuses from the uterine environment may result in more rapid degradation of antiviral proteins in vitro which could explain the apparently lower antiviral activity in conceptus culture medium compared to uterine flushings (Mirando et al., 1990). Secretion of protease inhibitors by endometrium may provide a system for regulation of antiviral proteins secreted by developing pig conceptuses. However, secretory products of the endometrium also decreased antiviral activity in conceptus culture medium. Inhibitory, as well as stimulatory, influences of the endometrium on secretion of proteins by pig conceptuses must, therefore, be considered.

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