Implantation potential and fetal viability of mouse embryos cultured in media supplemented with platelet-activating factor

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Summary. Supplementation of culture media with PAF failed to increase the proportion of 2-cell mouse embryos which developed through to the expanded blastocyst stage after 72 h of culture *in vitro*. Culture of embryos with 1.86 μM -PAF, however, significantly increased the number of cells in expanded blastocysts. Culture *in vitro* with 0.186 or 1.86 μM -PAF significantly increased the ability of embryos to implant upon transfer to pseudopregnant recipients. The specificity of this effect was demonstrated by its inhibition by the specific PAF-receptor antagonist, SRI 63-441. Of the embryos which implanted, there was no difference in the proportions of PAF-treated or control embryos which resorbed nor was there any effect of PAF on fetal weight or crown-rump length measurements. Embryos cultured with 1.86 μM -PAF did have slightly heavier placentae than their respective controls. These results demonstrate the ability of exogenous PAF to enhance the viability of preimplantation mouse embryos cultured *in vitro* and support the hypothesis that a major site of the action of PAF during early pregnancy is the embryo itself.

Keywords: embryo; PAF; implantation; culture *in vitro*; fetus; mouse

Introduction

Platelet-activating factor (PAF) is an ether phospholipid which is produced by mouse and human preimplantation embryos (O'Neill, 1985; Collier *et al.*, 1988). The production of PAF was greater for morphologically normal human embryos after in-vitro fertilization compared with those with defects such as fragmentation or uneven-sized blastomeres (O'Neill *et al.*, 1987; Collier *et al.*, 1988). Furthermore, the amount of PAF produced by human embryos was correlated with their pregnancy potential after transfer (O'Neill & Saunders, 1984; O'Neill *et al.*, 1987). The ability of mouse embryos to produce PAF, however, was shown to decrease with increasing time in culture (Ryan *et al.*, 1989).

It is generally accepted that culture of preimplantation-stage embryos *in vitro* is detrimental to their pregnancy potential following transfer (Bowman & McLaren, 1970a; Harlow & Quinn, 1982) although the precise reasons for this loss of viability have not been defined. It has been suggested that embryo-derived PAF activity is necessary for successful implantation (Spinks & O'Neill, 1988) and that its site of action is at the embryonic level (Ryan *et al.*, 1989; Ryan *et al.*, 1990; Spinks *et al.*, 1990). In view of the reduced secretion of embryo-derived PAF after in-vitro culture (Ryan *et al.*, 1989), PAF may be a limiting factor in maintaining embryo viability during culture *in vitro*. To test this hypothesis, this study examined the effects of supplementing culture medium with PAF on embryonic development rates *in vitro* and the ability of the embryo to implant and develop into normal fetuses after transfer.

Materials and Methods

Embryo production. Embryos were collected at the 2-cell stage from 6-week-old random-bred Swiss albino mice of the Quackenbush strain (Qs: Department of Veterinary Physiology, University of Sydney, NSW, Australia). Mice were induced to superovulate by the intraperitoneal injection of 10 i.u. PMSG (Folligon; Intervet, Boxmeer, The Netherlands) and 10 i.u. hCG (Chorulon; Intervet) given 48 h apart. At 42–44 h after hCG injection, the oviducts of mated mice were flushed with a Hepes-buffered HTF medium (Quinn *et al.*, 1985) to recover embryos. Flushing medium was supplemented with 3 mg bovine serum albumin (BSA)/ml (bovine albumin, crystallized; Miles Inc., Kankakee, IL, USA).

Embryo culture. Two-cell embryos were cultured for 72 h in bicarbonate-buffered Quinn's HTF medium (Quinn *et al.*, 1985) supplemented with 3 mg BSA/ml (Q + BSA). Embryos were cultured in groups of 20 in 20 µl droplets, following an overnight equilibration period, under heavy-weight liquid paraffin oil (BDH Chemicals Ltd, Poole, Dorset, UK) within plastic tissue culture dishes (Falcon, Becton-Dickinson Co., Rutherford, NJ, USA) in an atmosphere of 5% CO₂ in air at 37°C. Culture media were supplemented with 0, 0.186, 1.86, 9.3 or 18.6 µM-PAF (Sigma Chemical Co. St Louis, MO, USA). PAF was prepared as a 1.86 mM stock solution in chloroform and stored at –20°C. Aliquants were transferred to sterile glass siliconized tubes and dried under a continuous stream of N₂ and then solubilized at a concentration of 0.186 mM in Q + BSA. This solution was further diluted before addition to the culture media by 1:9 dilutions to give the desired final concentrations. Preliminary studies using [³H]PAF (Amersham, Bucks, UK) demonstrated no loss of activity from droplets of Q + BSA under paraffin oil during a 72-h culture period.

Effect of PAF on in-vitro embryo development. As a measure of embryo developmental potential *in vitro*, the proportion of 2-cell embryos, cultured in Q + BSA supplemented with 0, 0.186, 1.86, 9.3 or 18.6 µM-PAF, that reached the expanded blastocyst stage after 72 h was determined. The numbers of cells in embryos cultured from the 2-cell stage for 72 h in Q + BSA containing 0, 0.186 or 1.86 µM-PAF were also assessed. Embryos were fixed and stained according to a modification of the method of Roberts & O'Neill (1988). Briefly, embryos were exposed to sequential hypotonic solutions of 1% and 0.5% sodium citrate for 20 and 10 min, respectively. They were then transferred to a methanol:acetic acid (3:1 v/v) solution for a further 5–10 min. Groups of 5–10 embryos were then transferred to a clean slide and allowed to dry. Before complete evaporation, 1–2 drops of 60% acetic acid were added to facilitate embryo spreading. Slides were left at room temperature to dry and then stained with 5% Giemsa (Gurr's R66 Improved; BDH Chemicals, Poole, Dorset, UK) for 5 min, mounted and viewed at ×200 magnification to allow estimates of the numbers of cells to be made.

Effect of PAF on embryo viability. The viability of embryos cultured in media supplemented with PAF was assessed by monitoring their ability to implant and develop into fetuses after transfer: 2-cell embryos were cultured for 72 h in Q + BSA supplemented with 0 (control), 0.186 or 1.86 µM-PAF through to the expanded blastocyst stage and transferred to recipients on Day 3 of pseudopregnancy. Recipients were Quackenbush females of at least 8 weeks of age mated with vasectomized males.

The embryo transfer procedure used was that described by McLaren & Michie (1956). Each recipient had control embryos transferred to one uterine horn, chosen in a randomized manner, and PAF-treated embryos (0.186 or 1.86 µM) to the contralateral horn: 10 or 6 embryos were transferred per uterine horn to animals killed on Days 8 or 17 of pregnancy, respectively. The numbers of implantation sites were recorded on Day 8 and the numbers of fetuses and resorbing implantation sites (the sum of which equals the total number of implantations) on Day 17. Only data from those recipients which, at the time of necropsy, were identified to have at least one implantation site were included for presentation and analyses. Fetal and placental weights were also recorded, as were crown–rump length measurements for all recipients killed on Day 17.

To examine the specificity of the effect of PAF on embryo viability, a further experiment was undertaken utilizing the PAF receptor antagonist SRI 63-441 (Sandoz Research Institute, East Hanover, NJ, USA). Two-cell embryos were cultured for 72 h in Q + BSA supplemented with 0 (control), 1.51 µM-SRI 63-441 or 0.186 µM-PAF in addition to 1.51 µM-SRI 63-441. Expanded blastocysts were transferred to recipients in the same manner as described for the first transfer experiment. Necropsies were performed on Day 8 of pregnancy and the number of implantation sites recorded.

Statistical analysis. Data for the effect of PAF on the rate of embryo development during culture *in vitro* were analysed by χ^2 analysis. Cell numbers for expanded blastocysts after culture in media supplemented with different concentrations of PAF were analysed by analysis of variance with differences between means compared by the Newman–Keuls' test (Steel & Torrie, 1960). Implantation, fetal development and resorption site data from the transfer experiments were subjected to logistic regression analysis (Adena & Wilson, 1982). In fitting all models, an effect due to variability between recipient mice has been incorporated because two results, arising from the transfer of control embryos to one uterine horn and PAF-treated embryos to the contralateral horn, were available for each recipient. There was considerable variation between recipients in the number of fetuses recovered from the left and right uterine horns. Mean fetal and placental weights as well as crown–rump lengths were, therefore, calculated and provided data in a balanced design for subsequent analysis by Repeated-Measures analysis of variance (Winer, 1971).

Furthermore, as fetal and placental weights have been reported to be inversely related to litter size (McLaren, 1965), the number of fetuses was included as a covariate in the analysis to correct for its potentially confounding effect.

Results

Supplementation of media with 0.186 or 1.86 μM -PAF had no effect on the proportion of 2-cell embryos that developed to expanded blastocysts after 72 h culture *in vitro* (Table 1) but did cause a dose-dependent increase in the number of cells in expanded blastocysts (control: 73.1 ± 2.1 (110) vs 0.186 μM -PAF: 78.2 ± 2.4 (97) vs 1.86 μM -PAF: 81.9 ± 2.8 (79)). At a dose of 1.86 μM -PAF, an average increase of 12% in the number of cells compared with controls was observed ($P < 0.05$). A higher dose of PAF (18.6 μM) caused fewer embryos to develop through to the blastocyst stage after 72 h culture *in vitro* (Table 1, Exp. 1).

Table 1. Effect of PAF supplementation of culture media on in-vitro development of 2-cell mouse embryos

Exp.	Dose of PAF (μM)	No. of embryos cultured	No. of expanded blastocysts at 72 h (%)
1	0	235	159 (68)
	1.86	232	146 (63)
	9.3	248	138 (56)
	18.6	206	104 (50)*
2	0	3060	2338 (76)
	0.186	1772	1335 (75)
	1.86	1713	1248 (73)

*Value differs significantly from control (0 μM -PAF), $P < 0.01$.

In the experiment evaluating the effects of PAF on the pregnancy potential of embryos cultured *in vitro*, 18 replicates were conducted. Implantation data were available for 36 and 68 mice killed on Days 8 and 17 of pregnancy, respectively. The culture of embryos in media supplemented with 0.186 or 1.86 μM -PAF significantly ($P < 0.001$) increased the number of embryos that implanted compared with their respective controls (Table 2). There was considerable variability between replicates, however, in the extent to which PAF supplementation of media caused enhanced rates of implantation. Figure 1 shows the results presented in Table 2 when sorted by implantation rate for control embryos. When the implantation rate of control embryos was low ($\leq 50\%$), the culture of embryos in media supplemented with PAF resulted in increased rates of implantation whereas when control rates were high ($> 50\%$), no effect of PAF was observed.

Necropsies of animals performed on Day 17 allowed calculation of both the proportion of transferred embryos resulting in viable fetuses and the proportion that implanted but subsequently resorbed. There was no effect of PAF, at either dose, on the proportion of implanted embryos which resorbed (0.186 μM -PAF: 30/100 (30%) vs control: 26/86 (30%), n.s.; 1.86 μM -PAF: 29/111 (26%) vs control: 23/91 (25%), n.s.). The effects of PAF on fetal development rates therefore reflected those observed on implantation rates although the differences failed to reach significance (0.186 μM -PAF: 70/186 (38%) vs control: 60/186 (32%), n.s.; 1.86 μM -PAF: 82/222 (37%) vs control 68/222 (31%), n.s.).

PAF (0.186 or 1.86 μM) had no effect on fetal weights or on crown-rump lengths (Table 3). However, at a dose of 1.86 μM -PAF, placental weights were significantly ($P = 0.03$) heavier than those in their respective controls.

Fig. 1. Implantation rates of control (solid bars) and PAF-treated (0.186 and 1.86 μM combined, hatched bars) mouse embryos assessed on the basis of whether the implantation rate of control embryos for a given replicate was 0–25, 26–50, 51–75 or 76–100%. Number of embryos transferred in parentheses. *Significantly different from controls, $P < 0.001$.

Table 2. Implantation rates of mouse embryos cultured in the presence of 0 (control), 0.186 or 1.86 μM -PAF

Dose of PAF (μM)	No. of pregnant mice	Implantation rate* (%)	
		Control	PAF
0.186	51	168/386 (44)	227/386 (59)†
1.86	53	177/382 (46)	218/382 (57)†

*Implantation rate = no. of implantations/no. of embryos transferred.
†Significantly different from controls, $P < 0.001$.

Culture of embryos with SRI 63-441, a PAF receptor-antagonist, was performed to assess the specificity of the apparent effect of PAF in increasing their implantation potential. Culture of embryos in media supplemented with 1.51 μM -SRI 63-441 or 1.51 μM -SRI 63-441 together with 0.186 μM -PAF had no effect, compared with control embryos, on the proportion of 2-cell embryos that developed to the expanded blastocyst stage (control: 428/518 (83%) *vs* SRI 63-441: 217/249 (87%) *vs* SRI 63-441 + PAF: 217/265 (82%); $\chi^2 = 3.20$, $P = 0.2$). Culture in the presence of 1.51 μM -SRI 63-441 had no effect on the rate of implantation following transfer (Table 4, $P = 0.1$). However, supplementation of media with 1.51 μM -SRI 63-441 in addition to 0.186 μM -PAF resulted in a significant ($P = 0.01$) decrease in the implantation rate (Table 4).

Table 3. Fetal and placental weights and crown-rump length measurements on Day 17 of pregnancy of embryos cultured in the presence of 0 (control), 0.186 or 1.86 μM -PAF (mean $\pm$ s.e.m.)

Treatment	No. of pregnant mice	Fetal wt (mg)	Placental wt (mg)	Crown-rump length (mm)
Control-A	25	646 $\pm$ 46	200 $\pm$ 7	17.1 $\pm$ 0.4
0.186 μM -PAF	26	702 $\pm$ 51	203 $\pm$ 8	17.5 $\pm$ 0.6
Control-B	24	732 $\pm$ 32	191 $\pm$ 5	17.8 $\pm$ 0.4
1.86 μM -PAF	30	710 $\pm$ 36	206 $\pm$ 5*	17.9 $\pm$ 0.3

*Significantly different from control, $P = 0.03$.

Table 4. Implantation potential of mouse embryos cultured in the presence of PAF and the PAF antagonist SRI 63-441

Treatment		No. of pregnant mice	Implantation rate* (%)
PAF (μM)	SRI 63-441 (μM)		
0	0	9	41/90 (46)
0	1.51	9	50/90 (56)
0	0	13	63/130 (48)
0.186	1.51	13	46/130 (35)†

*Implantation rate = no. of implantations/no. of embryos transferred.

†Significantly different from controls (no PAF or SRI 63-441), $P = 0.01$.

Discussion

A direct effect of PAF on the preimplantation mouse and human embryo was suggested by the observations that the addition of PAF during culture enhanced the oxidative metabolism of glucose and lactate (mouse: Ryan *et al.*, 1989; Ryan *et al.*, 1990; human: O'Neill *et al.*, 1989) and PAF antagonists reduced mouse blastocyst outgrowth *in vitro* (Spinks *et al.*, 1990). The treatment of embryo donors before embryo transfers, but not recipients, with a specific PAF-antagonist reduced implantation rates, suggesting that the antagonist was acting at the embryonic level (Spinks *et al.*, 1990). The secretion of PAF by embryos is highly variable (O'Neill & Saunders, 1984; O'Neill *et al.*, 1987) and decreases with increased time in culture (Ryan *et al.*, 1989). The present study shows that supplementation of culture media with exogenous PAF increased the implantation potential of embryos cultured *in vitro*. Similarly, we have also shown an increase in pregnancy rates of human embryos produced by in-vitro fertilization after culture in PAF-supplemented media (O'Neill *et al.*, 1989).

Development of 2-cell mouse embryos through to the blastocyst stage during culture *in vitro* takes 18–24 h longer and the resulting blastocysts have fewer cells than do embryos cultured *in vivo* (Bowman & McLaren, 1970b; Harlow & Quinn, 1982). The times taken for the total cell number to double during culture from the 2-cell to blastocyst stages were estimated at 10 h and 17 h for embryos cultured *in vivo* and *in vitro*, respectively (Harlow & Quinn, 1982). Although increased cleavage rates *in vitro* have been reported to result from the reduction of the oxygen tension during

culture from 20% to 5% (Quinn & Harlow, 1978) and by supplementing media with progesterone (Roblero & Izquierdo, 1976), it is still generally accepted that the *in-vitro* culture conditions presently available are far from optimal. The addition of PAF to culture media in the present study did not increase the rate of development as assessed on morphological criteria but a dose of $1.86 \mu\text{M}$ -PAF did result in a modest increase in the rate of cell division.

The effect of PAF in enhancing the implantation potential of embryos cultured *in vitro* appeared to be related to the pregnancy potential of the corresponding control embryos in each replicate of the experiment. When control embryos had a high pregnancy potential, exogenous PAF had little or no beneficial effect while the addition of PAF had a significant effect when the implantation capacity of control embryos was poor (Fig. 1). It may be that in these latter cultures the secretion of embryo-derived PAF was low, resulting in embryos of poor pregnancy potential, a situation which was alleviated with the addition of exogenous PAF. The logistics of our experimental design did not allow us to test this hypothesis in the present study. However, we have previously shown that there was a positive correlation between the quantity of PAF produced by human embryos derived from *in-vitro* fertilization and their subsequent pregnancy potential (O'Neill & Saunders, 1984; O'Neill *et al.*, 1987; Collier *et al.*, 1988).

Culture of embryos in media supplemented with the specific PAF receptor-antagonist, SRI 63-441 (Handley *et al.*, 1986), inhibited the beneficial effect of exogenous PAF on the implantation rate. Indeed, it caused a significant suppression compared with controls. Similarly, SRI 63-441 significantly inhibited the direct effects of exogenous PAF on the oxidative metabolism of lactate and glucose by 2-cell mouse embryos during culture *in vitro* (Ryan *et al.*, 1990). No effect of SRI 63-441 on the implantation potential of cultured embryos was observed in the absence of exogenous PAF. Possible explanations for this observation include that the antagonist, at the concentration used, may be acting as a partial agonist or that embryo-derived PAF and exogenous PAF act on or are made available to the embryo by different mechanisms. It is apparent, however, that the dynamic relationships between exogenous PAF, embryo-derived PAF and PAF antagonists require extensive investigation at the cellular level if we are to understand better the mechanisms responsible for the contragestational actions of antagonists (Spinks & O'Neill, 1988) and the observed effect of exogenous PAF in enhancing the implantation potential of cultured embryos.

Culture of preimplantation embryos *in vitro* has been shown to depress fetal growth, but not placental growth (Bowman & McLaren, 1970a; Harlow & Quinn, 1979). This differential effect of culture on fetal and placental weights led to the suggestion that the inner cell mass is more sensitive to sub-optimal culture conditions than is the trophoctoderm (Bowman & McLaren, 1970a). Furthermore, the rate at which cells of the inner cell mass divide was greater than the rate of division of trophoctoderm cells for embryos developing *in vivo* whereas the opposite was demonstrated for embryos cultured *in vitro* (Harlow & Quinn, 1979). The significance of the increased placental weight of embryos cultured in media supplemented with $1.86 \mu\text{M}$ -PAF while having no effect on fetal weight is unclear. It may be that the increased cell numbers of expanded blastocysts cultured in the presence of PAF reflects an effect of PAF specifically on trophoctoderm cells.

In conclusion, the present study demonstrates that exogenous PAF has the ability to increase the implantation potential of embryos cultured *in vitro* but has no substantial effects on subsequent fetal development. Furthermore, it supports the hypothesis that PAF is important during the preimplantation stage of development in the mouse and that a major site for its actions is the embryo itself.

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